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Relationship between Brucella melitensis and the associated immune response after intranasal infection in mice

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Relationship between *Brucella melitensis* and the associated immune response after intranasal infection in mice

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By Delphine Hanot Mambres

Summary

Brucella melitensis is a facultative intracellular bacterium responsible for one of the most common zoonosis in the world, mainly transmitted by oral ingestion of contaminated food and aerosol. Infected humans usually have an undulant fever that can become a chronic disease if it is not treated. Abortion and sterility are the major symptoms in domestic and wild animals. The classical intraperitoneal (i.p.) model of infection in mice has been useful for discovering virulence factors of *Brucella* and characterizing effectors of protective host immunity. But this model is not representative of the natural route of infection and much remains unknown regarding the immune response and mechanisms developed by *Brucella* to persist in the host. Consequently, we decided to develop an intranasal (i.n.) model of infection to study the immune response of mice against *Brucella melitensis* infection.

In the first part of the study, using wild type and genetically deficient C57BL/6 mice, we analyzed the nature and the function of immune cellular populations controlling *Brucella* infection, including the action of specific components of mucosal immunity. We confirmed the crucial role of IFN-γ-producing CD4 Th1 cells in the control of the primary i.n. infection by *Brucella melitensis*. We also observed an early role for γδ T lymphocytes and production of IL-17A in the control of the bacterial load in lungs. We showed that B lymphocyte deficiency does not affect the efficiency of the primary or secondary immune response against *Brucella*.

In the second part of the work, using susceptible IL12p40-deficient BALB/c mice, we characterized the preferential cellular niche for *Brucella* in the spleen during the persistent phase of infection. We confirmed that *Brucella melitensis* exhibits a tropism for the myeloid lineage in susceptible IL12p40-deficient BALB/c mice. The large majority of infected cells expressed CD11c and CD205 markers, which are specific for dendritic cells. But they are lipid-rich and they display high levels of arginase1, a typical marker of IL-4-induced alternatively activated M2 macrophages. To determine if these cells are Th2-induced M2 macrophages, we analysed the impact of IL-4/STAT6 signaling deficiency on the course of infection and the phenotype of infected cells in IL12p40-deficient mice. We observed no differences between IL-12p40 and IL-12p40/STAT6 deficient mice, demonstrating that infected cells are not dependent on IL-4 and cannot be considered as alternatively activated M2 macrophages induced by the Th2 response.

In conclusion, this work provides a better understanding of the immune response induced by *Brucella melitensis* in an i.n. model of infection, and describes, for the first time, the phenotype of a preferential cellular niche of the pathogen in susceptible mice during the chronic phase of infection.

You will never do anything in the world without courage. It is the greatest quality of the mind next the honor.
Aristotle

ABBREVIATIONS

AAM/M2	Alternatively activated macrophage
AID	Activation Induced cytidine deaminase
Ab	Antibody
Ag	Antigen
APC	Antigen presenting cell
Arg1	Arginase1
ATP	Adenosine tri-phosphate
BALT	Bronchus associated lymphoid tissue
BCR	B cell receptor
BCV	Brucella containing vacuole
BMDM	Bone marrow derived macrophage
BTP1	Brucella TIR containing protein
CAM/M1	Classically activated macrophage
CARKL	Carbohydrate kinase-like
CCR2	Chemokin receptor 2
CD	Cluster of differentiation
CFU	Colony forming unit
CpG	Cytosine phosphate guanosine
DALIS	Dendritic cells aggresome-like induced structures
DAMPS	Damage associated molecular patterns
DC	Dendritic cell
DC SIGN	Dendritic cell- specific icam3-grabbing non integrin
DOK2	docking protein 2
DTH	Delayed-type hypersensitivity
EE	Early endosome
ER	Endoplasmic reticulum
ERK	extracellular signal-regulated kinase
FA	Fatty acid
FasL/R	Fas ligand/receptor
FIZZ	Found in inflammatory zone
Fpn	Ferroportin

i.n.	Intranasal
i.p.	intraperitoneal
IC	Immune complex
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
INOS	Inducible nitric oxyde synthase
JAK1	Janus kinase 1
JNK	JUN N-terminal kinase
LE	Late endosome
LN	Lymph node
LPS	Lipopolysoccharide
MALT	Mucosal associated lymphoid tissue
MAPK	Mitogen-activated protein kinase
MARCO	Macrophage receptor
MD2	Myeloid differenciation 2
MHC	Major histocompatibility complex
MLN	Mesenteric lymph node
MMM	Metallophilic macrophage
MOMA	Monocyte/Macrophage
MR	Mannose receptor
MuMT	µM mutation (B cell deficient mice)
MyD88	Myeloid differentiation factor 88
MZ	Marginal zone
MZM	Marginal zone macrophage
NFκB	activation of nuclear factor-κB
NK	Natural Killer
NKT	Natural Killer T (cell)
NLR	Nod like receptor
NO	Nitric oxyde
NOD	Nucleotide oligomerization domain
NOHA	*N*G-hydroxy-L-arginine
NRAMP1	Natural resistance associated macrophage protein 1

OAT	Ornithine aminotransferase
OCR	Oxygen consumption rate
ODC	Ornithine decarboxylase
Omp	Outer membran protein
PAMP	Pathogen associated molecular pattern
PPAR	Peroxisome proliferator activated receptors
PPP	Pentose phosphate pathway
PRR	Pattern recognition receptor
PTX	Pentaxin
r.p.	red pulp
RAG	Recombination associated gene
RASA2	RAS GTPase-activating protein
RhoA	Ras homolog gene family, member A
RLR	Rig like receptor
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SCV	Salmonella containing vacuole
SH domain	Small hydrophobic domain
SIRPα	signal-regulatory protein-α
SLAM	Signaling lymphocytic activation molecule
SMAD	Sma and mad related proteins
SOCS 3	suppressor of cytokine signaling 3
SP	Pulmonary surfactant-associated protein
STAT6	Signal tranducer and activator of transcription 6
SR	Scavenger Receptor
ssRNA	single strand RNA (Ribonucleic acid)
TAP-1	Transporter associated with antigen procession
TcpB	TIR domain containing protein
TCR	T Cell Receptor
TGF	Transforming growth factor
Th	T helper
TIR	Toll/Interleukin-1 Receptor
TLR	Toll like receptor

TNF	Tumor necrosis factor
Treg	T regulator
TREM2	Triggering receptor expressed by myeloid cells 2
TRIF	Toll/Interleukin-1 Receptor domain-containing adapter inducing IFNβ
w.p.	white pulp
WT	Wild type

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TABLE OF CONTENTS

INTRODUCTION

1. Foreword: Relationship between microorganisms and their hosts

Host-parasite relationship is a phenomenon that appeared on earth at the same time as life emerged around 3.8 billion years ago. The evolution is based on constant intra and inter-specific interactions in which each partner devotes its life to meet its own needs and to replicate. It gave rise to extremely diversified and dynamic interactions between microorganisms and their hosts. These relationships can lead to peaceful coexistence (mutualism), as it is the case for the intestinal flora and its host, but it is far from being always the case: microorganisms can prosper to the detriment of their host. They are termed "pathogen" and generally induce damage to their host (parasitism) (Montgomery & McFall-Ngai, 1994). It is noteworthy that an invasion by a pathogen can also result in a chronic or a latent infection without detectable clinical symptoms, but following any changes in the environment, the host or the parasite can modify the equilibrium and the disease can take place (Araújo *et al.*, 2013). The battle between host and parasite refers to the "Red Queen's hypothesis", derived from the animation Alice in Lewis Carroll's "Through The Looking Glass", and used by Leigh Van Valen in 1973 to illustrate the law of extinction and co-evolution between competing species. It proposes that organisms continuously have to adapt, evolve and proliferate to have reproductive advantage and survive against competitor organisms in a changing environment (Van Valen, 1973). The outcome of the relationship will depend on the competition of the host's immune system and the escape mechanisms of the pathogen (Araújo *et al.*, 2013).

This thesis will focus on the pathogenic aspect of chronic bacterial infection, and the immune defenses implemented by animals. We are particularly interested in the pathogen *Brucella melitensis* and its ability to deal with the immune responses of mice.

2. *Brucella, the agent of brucellosis*

2.1. Historical overview of the pathogen

Skeletal remains from the Ancient Albanian city from 10th to 13th centuries AD revealed circular lytic lesions on the thoracic and lumbar vertebrae. They are similar to lesions from present pathologies known to affect these skeletal regions such as tuberculosis or brucellosis. Genetic screening and DNA sequencing revealed the presence of *Brucella* DNA and suggests that brucellosis has been present in this region at least since the Middle Ages (Mutolo *et al.,* 2012). Before this period, clue of the presence of brucellosis stays indirect. In August 79 AD, a catastrophic volcanic eruption of Mount Vesuvius was responsible for the sudden death of thousands of Roman residents. Examination of the skeletons revealed typical lesions of brucellosis in more than 17 % of the inhabitants. It is consistent with the fact that the population had the habit to consume sheep's milk and its derivatives. A carbonized cheese found in the ruins reinforced this conclusion when it revealed the presence of coccobacilli-like forms morphologically and dimensionally similar to *Brucella* (Capasso, 2002). The examination of Egyptian bones that revealed osteoarticular lesions is a clue that brucellosis was present in Africa around 750 BC (Pappas & Papadimitriou, 2007). More recently, scientists have analyzed partial skeleton of an Australopithecus from the hominid side of Sterkfontain South Africa (*Australopithecus africanus*). The lesions are consistent with the one developed in case of brucellosis. It would coincide with the hypothesis that *Australopithecus africanus* began to include meat in its diet and it moves back the potential origin of brucellosis to about 2.5 million years ago. (D'anastio et al., 2009).

One became aware of the disease in Malta during the Crimean War in 1850 for the first time. And it is only in 1887 that David Bruce identified *Micrococcus melitensis* as the pathogen responsible for the "Malta fever" in a British soldier who died from the disease. The genus "*Brucella*" was given in honor of David Bruce when a close species was discovered in cattle. But the zoonotic nature of Malta fever was only discovered in 1905 by isolating *Brucella melitensis* from goat's milk (Seleem *et al.*, 2010).

Table 1 : Currently described *Brucella* species and host associated.	
Species	**Preferential host**
B. melitensis	Sheep, goats, camels
B. abortus	Cattle, buffalo, elk, yaks, camels
B. suis	Domestic pigs, wild boar, reindeer, caribou, rodents
B. canis	Canines
B. ovis	Sheep
B. neotomae	Rodents
B. ceti	Porpoises, dolphins, whales
B. pinnipedialis	Seals
B. microti	Red foxes, common voles (also isolated from soil)
B. inopinata	Unknown
B. papionis	Baboons

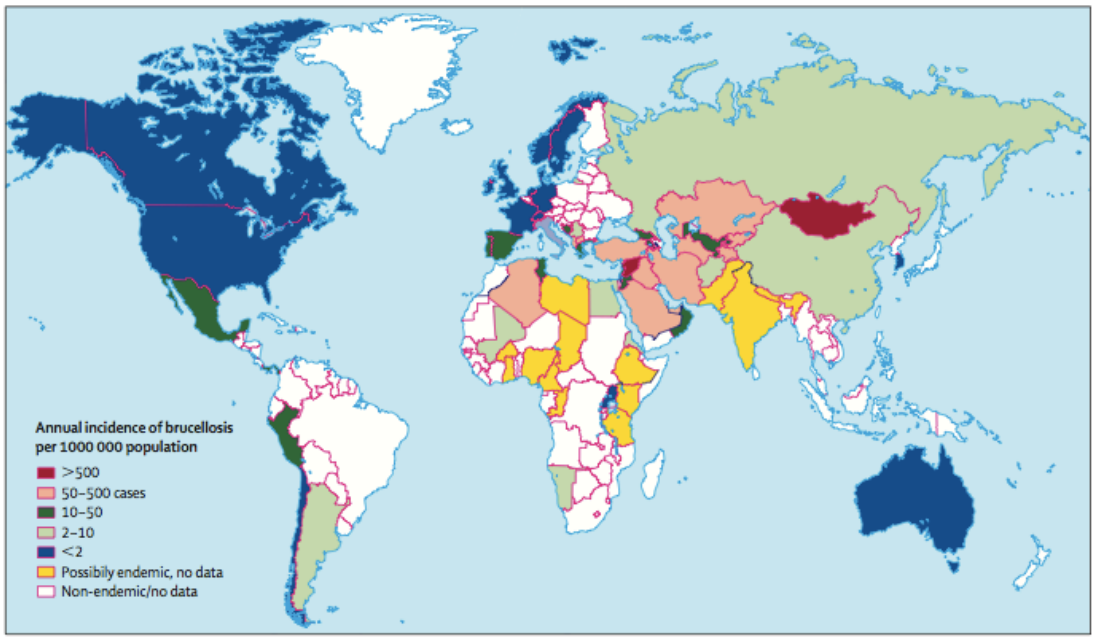


Figure 1: Worldwide incidence of Brucellosis between 2000 and 2006 (source: Pappas et al., 2006).

2.2. Epidemiology in humans

To date, 11 species of *Brucella* have been identified (**Table 1**), but it seems that 6 species are able to generate a disease in humans (*B. melitensis, B. abortus, B. suis* and, to a lesser extent, *B. canis, B. ceti and B. pinnipedialis*). Brucellosis is one of the most common bacterial zoonosis in the world with more than 500 000 new reported cases each year. However, epidemiological data underestimate the disease incidence for various reasons, such as barriers to accessing health care and misdiagnosis. Global distribution of the disease has changed over the last decade and is still in constant evolution, due to sanitary, socio-economic and political reasons (Pappas *et al.*, 2006). **Figure 1** shows the most recent representation of the worldwide incidence of brucellosis (between 2000 and 2006) (Pappas *et al.*, 2006). The disease is eradicated in many developed countries but remains highly prevalent in the developing world. Central and South America, The Middle East, Africa and west of Asia are the most affected regions in the world (Dean *et al.*, 2012; Medzhitov, 2007; Pappas *et al.*, 2006).

2.3. Pathology and transmission

Human brucellosis is mainly associated with an undulant fever during the acute phase. If the disease is not treated, human can suffer from multiple complications, depending on the site of infection. Direct cutaneous contact, ingestion of animal products and inhalation of airborne agents are the main routes of transmission in humans. (Acha & Szyfres, 2001). Encephalitis, meningitis, spondylitis, arthritis, endocarditis, orchitis, prostatitis or even abortion can be the symptoms of the chronic disease in humans (Acha & Szyfres, 2001).

The pathology in animals is mainly characterized by abortion in pregnant females but males can also become sterile. Most of the females abort only once even if the placenta is highly infected in subsequent normal calvings (Morgan, 1969; Saenz *et al.*, 2008). It seems that 60 to 70 % of the fetuses born to infected mothers carry the disease (Díaz Aparicio, 2013). Horizontal transmission of the disease is frequent and is usually ensured by inhalation of the bacterium during the birth, or ingestion of food or water contaminated with secretion or aborted fetal remains from infected animals. Juveniles are often infected by contaminated milk. Milking healthy females after the infected ones with the same teat cups is also a cause of contamination in farms (Díaz Aparicio, 2013). In countries where brucellosis

is endemic, animal production is disturbed because of the reduction of milk production, the abortion and delayed conception. The slaughter of contaminated animals and the decrease in productivity generates extensive economic losses estimated to several hundreds of million dollars (Seleem *et al.*, 2010).

Brucella species are classified according to their preferential host but cross-infections can also occur between species. For example, *Brucella abortus*, the etiologic agent for bovine brucellosis, can infect goats that share pasture and facilities with cattle, or dogs coming from the same farm (E. Diaz Aparicio, 2013; B. K. Baek *et al.*, 2003). *Brucella melitensis*, normally associated with infection in goats and sheep, can also be responsible for brucellosis in dogs, cattle and camels (Acha & Szyfres, 2001). Cross-infection can become an important issue, like in The Middle East where *B. melitensis* regularly infects cattle while it is the most frequently reported species infecting human (Corbel, 2006).

2.4. Diagnosis of Brucellosis

Diagnosis of brucellosis in humans can be achieved by the isolation of *Brucella* from samples such as blood, cerebrosplinal or synovial fluid. Unfortunately, the detection of the pathogen in the sample is far from being a frequent success. Serological tests are therefore often necessary for the diagnosis of the infection. In Belgium, CODA-CERVA is the national reference laboratory for brucellosis in humans. Two serological tests are done: (I) Rose Bengal test, based on the agglutination of the serum in presence of *Brucella* antigen, and (II) ELISA (Enzyme-Linked Immunosorbent Assay), which reveals the presence of anti-LPS antibodies from the serum by an enzymatic reaction. The combination of the two tests is required because their sensitivity is not optimal. It is also important to know that cross-reaction with other pathogens like *Yersinia enterolitica* or *Francisella tularensis* is possible. In Belgium, brucellosis is rare in humans: there are only 5 to 10 cases of brucellosis per year and patients are generally allochtons, coming from countries where brucellosis is endemic. Therefore, if a patient has, for example, an undulent fever, diagnosis can take times before thinking about brucellosis.

Brucellosis is not endemic in livestock in Belgium but the presence of the bacterium in wildlife is not excluded. Its transmission to the livestock is therefore possible and surveillance is necessary to avoid a new epidemy in the country. The main clinical sign of

brucellosis in animals is abortion at the first gestation. In case of abortion in bovine, bacteriology is systematically made. if *Brucella* is responsible for the event, it is detected without any problem. However, serological tests are essential if there is no symptoms. In Belgium, seroagglutination test (SAT) and commercial ELISA are done in regional laboratories. If the sample is positive, it is sent to the CODA-CERVA where a more effective non-commercial ELISA test is done to confirm the results before make a decision about the animal.

2.5. Treatment and vaccination

Contaminated humans have to be treated by antibiotics to avoid chronic complications. The intracellular localization of *Brucella* and its ability to replicate in cells specialized in the destruction of pathogens explain that treatment failure and relapse rate are relatively high and depend on drug combination and patient compliance. A combination of two antibiotics is necessary to avoid relapse and to prevent multiplication of intracellular bacteria. Presently, the Food and Agriculture Organisation of the United Nation and World Health Organization (FAO/WHO) Expert Committee consider the combination of high doses of doxycycline with rifampicin during six weeks as the optimal treatment (Seleem *et al.*, 2010).

Infected animals are not treated; they have to be eliminated. In countries where the zoonosis is endemic, vaccination of healthy livestock is important to limit the infection from contaminated ones and wildlife: “Strain 19” is the vaccine with proven efficacy against bovine brucellosis, and the vaccine “Rev1” controls the ovine and caprine brucellosis. Nevertheless, these live attenuated vaccines are not fully effective; they can trigger abortion and be infectious for humans. Moreover, “Rev1” is resistant to streptomycin, which is an antibiotic that could be used in the treatment of brucellosis, and induces antibodies that interfere with diagnosis of the real disease (Moriyón *et al.*, 2004). Consequently, an effective vaccine that would (i) not interfere with diagnosis, (ii) not induce abortion, (iii) not resist to antibiotics used for treatment and (iv) would be safe enough for human still has to be found.

TABLE 2: PRRs of the innate immunity and their PAMPS		
	PRRs	**PAMPs**
Transmembran PRRs	Toll-Like Receptors	
	TLR1	Peptidoglicans, lipoproteins
	TLR2	Lipoproteins, glycolipids, Fimbriae
	TLR4	Lipopolysaccharide (LPS), Fimbriae, pili
	TLR5	Flagellin
	TLR6	Diacyl lipoproteins
	TLR7/8	ssRNA
	TLR9	unmethylated CpG-DNA
	Scavenger receptors	
	SR-AI/II	LPS, Lipoteichoic acid (LTA), CpG DNA
	MARCO	
	SR-B	
	C-type lectins	
	Mannose receptor	LPS, fucose, capsular polysaccharides, liparabinomannan (ManLam)
	DC-Sign	High mannose, fucose
	Dectin-1	B1,3-glucan
Cytosolic PRRs	Nod-Like Receptors	
	NOD1	diaminopimelic acid (DAP)
	NOD2	Muramyl Dipeptide (MDP)
	NALP1	MDP
	NALP3	RNA, DNA
	IPAF	Flagellin
Secreted PRRs	Collectins Ficolins Pentraxins	Carbohydrates (e.g. bacterial capsules)

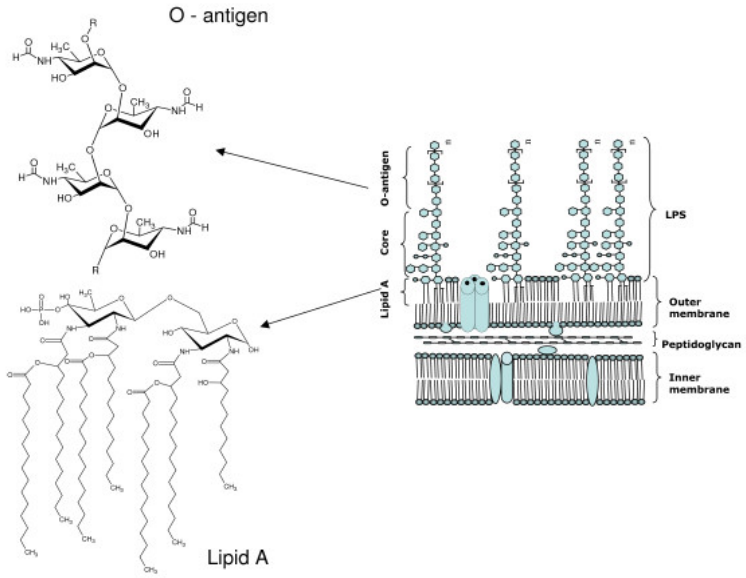


Figure 2: Schematic structure of LPS from *Brucella***.** It is composed of three domains : a polysaccharidic O chain in « smooth » strains, a core oligosaccharide and a lipid A. (source: Cardoso et al., 2006)

3. *Brucella and the immune response*

3.1. Innate immune vigilance facing *Brucella*

Classically, the innate immune system is able to recognize an invading pathogen through specific signatures: the pathogen associated molecular patterns (PAMPS). This recognition is mediated by pattern-recognition receptors (PRRs) **(Table 2).** The consequence of this recognition is the inflammation and the development of an immune response to clear the infection through the activation of effector mechanisms deleterious for the pathogen (for more details see **Box 1**). However, some pathogens, as *Brucella,* have developed strategies to escape and resist to the immune response (Gorvel, 2008). *Brucella* has developed furtive characteristics by losing, modifying or hiding PAMP-bearing molecules, ensuring low stimulatory activity of host cells:

- The bacterium does not display many classical structures involved in virulence such as pili, fimbriae and capsules. The absence of these PAMP-bearing molecules limits the detection of the pathogen by immune effectors (Martirosyan *et al.*, 2011).
- The unconventional structure of *Brucella* lipopolysaccharide (LPS) molecule (**Figure 2**) protects the bacterium from the innate immunity. The lipid A displays a low number of anionic groups, leading to a reduced binding of bactericidal cationic peptides. The long O-chains of LPS in smooth species (such as *B. melitensis, B. suis, B. abortus*) provoke steric barriers that contribute to the protection against bactericidal products and prevent deposition of complement at the surface of *Brucella* (Lapaque *et al.*, 2005). Moreover, the structure of *Brucella* LPS has reduced endotoxic properties because of a reduced TLR4 agonist activity, which is expected to stimulate neutrophils, monocytes and macrophages to produce adhesion molecules and pro-inflammatory cytokines (de Jong *et al.*, 2010; Ko & Splitter, 2003).
- Other molecules of the cell envelope, such as phospholipids, lipoproteins and ornithine-containing lipids also display reduction of negative charges and are weak inducers of pro-inflammatory molecules (such as Tumor Necrosis Factor (TNF)α, Interleukin (IL)-1β and IL-6) (Barquero-Calvo *et al.*, 2007; Gorvel, 2008).

Box 1: overview of the innate immune system

The innate immune system is composed of soluble effectors, such as the complement, and a diverse set of cells of haematopoeietic origin, including tissue-residing cells (macrophages and dendritic cells) and cells that patrol throughout the body via blood and lymphatic vessels (neutrophils, eosinophils and monocytes). The system constitutes the first line of the immune defense: innate cells are rapidly activated in contact of pathogens thanks to receptors called pattern-recognition receptors (PRRs) that recognize highly conserved foreign structures called pathogen-associated molecular patterns (PAMPs). **Table 2** enumerates PRRs that recognize bacterial PAMPs (from Doughty, 2011; Takeuchi & Akira, 2010).

The reaction of innate immunity is particularly rapid, but the response is not specific and the repertoire of PRRs is limited and invariant. This global system lacks long term and specific memory and does not have any ability to enhance the response in case of a second contact with the foreign structure. However it is noteworthy that some cell types such as Natural Killer (NK) cells and monocytes/macrophages, classified as innate immune cells, display a form of memory (termed trained immunity) against some antigens (Kleinnijenhuis et al., 2012; Quintin et al., 2012; Sun *et al.*, 2014).

Three major effector mechanisms can be attributed to the innate immune response against a pathogen:

- Phagocytosis: phagocytic cells engulf and internalize the pathogen in order to destroy it by a set of digestive enzymes and reactive oxygen species, among others.
- Inflammation: damaged tissues and resident immune cells secrete chemokines that attract phagocytes from the circulation to the infected site. Activated resident cells and phagocytes produce soluble mediators called cytokines and interleukins that further increase the phagocytic function of the cells. Increasing secretion of chemokines and cytokines leads to the recruitment of cells and plasma proteins in tissues through increased vessel permeability, triggering the classical signs of inflammation (swelling, redness, pain and heat). The inflammatory response is also essential for the healing process of the injured tissue. (Bellanti, 2012; Moser & Leo, 2010).
- Cytotoxicity: cytotoxic cells, such as NK cells, recognize infected cells and use a combination of mechanisms to lyse them. These include (i) the release of cytotoxic molecules such as perforin, which polymerizes to form a pore in target membrane, and granzymes, which are serine proteases that use the pore to reach the cytoplasm of target cell, (ii) Fas ligand (FasL), constitutively expressed or induced by interaction with aberrant cells, and (iii) membrane bound or secreted cytokines Tumor Necrosis Factor (TNF)α. These mechanisms activate the apoptosis, a non-inflammatory programmed cell death (Topham & Hewitt, 2009).

When the defenses of the innate immunity are not sufficient to destroy the invader, the efficiency of the response is improved by the activation of the adaptive immune response. Globally, after the contact and the recognition of the pathogen, the innate immune cells, and more particularly professional antigen-presenting cells (APCs), can migrate to secondary lymphoid organs and present antigens associated with a major histocompatibility complex (MHC) molecule to T lymphocytes. The antigen presentation, added to co-receptors and soluble signals, activates T lymphocytes and initiate the adaptive immune response (Medzhitov, 2007). For more details on the adaptive immune response see **Box 3 and 4**.

- Usually, flagellin is recognized by TLR5. In *Brucella*, its amino acid sequence is not well detected by this receptor because it does not have its agonist domain. Nevertheless, its recognition by at least the NOD-like receptor IPAF limits the persistence of *Brucella* in mice (Terwagne *et al.*, 2013).

Thanks to the characteristics listed above, *Brucella* is able to partially escape the innate immune system and reduces immuno-stimulatory properties of dendritic cells (DCs). Other mechanisms actively suppress DCs both at the cytokine production level (such as TNFα) and at the antigen processing and presentation level (de Jong *et al.*, 2010; Salcedo *et al.*, 2008) :

- Btp1 (*Brucella* Tir-containing protein) in *Brucella abortus* (homologous of TcpB in *Brucella melitensis*) is a bacterial protein with significant sequence similarity to Toll/interleukin receptor (TIR) domain family which interferes with the signaling pathway of TLR2 and TLR4. Thanks to this particularity, it is responsible for the decrease of the maturation of *Brucella*-infected DCs: it reduces the expression of DCs maturation marker such as the Cluster of Differentiation CD80 and DALIS (dendritic cells aggresome-like induced structures), the production of TNFα, IL-12 and Interferon (IFN) β, and the level of surface expression of MHCII (Cirl *et al.*, 2008; Salcedo *et al.*, 2008).
- The atypical *Brucella* LPS is recycled to the cell surface and forms stable macrodomains that sequester MHCII molecules. The complex between LPS and MHCII is non-functional and is not able to stimulate T lymphocytes properly (Forestier *et al.*, 2000).
- *Brucella* is also responsible for retention of MHCI in the Golgi apparatus, decreasing the activation of cytotoxic lymphocytes by the innate immunity (Barrionuevo *et al.*, 2012).

Box 2: Brucella trafficking in its host cell

Following phagocytosis, even if the host cell kills most of the *Brucella*, a small percentage of bacteria survive and replicate. To succeed, the pathogen controls the trafficking and maturation of its *Brucella* containing vacuole (BCV). The BCV starts normal trafficking with a transient association with early endosome. Next, the presence of markers such as LAMP1, Rab7 and Rab interacting lysosomal protein (RILP) on the BCV suggests that its trafficking undergoes temporary interactions with late endosome and even lysosome (Starr *et al.*, 2008). These fusions are accompanied with an increased acidification that turns out to be essential for intracellular surviving of *Brucella* (Porte *et al.*, 1999). Then, the trafficking of the vacuole becomes atypical for the host cell because the BCV begins to interact with endoplasmic reticulum (ER). There, it acquires ER markers including chaperones calreticulin, calnexin and the translocator sec61. The ER-like compartment containing *Brucella* constitutes the preferential location that ensures its replication and its virulence (Martirosyan *et al.*, 2011). The control of intracellular trafficking and the maturation of the phagosome is essential for the pathogen and involves specific *Brucella* factors, including virB type IV secretion system (Comerci *et al.*, 2001; Delrue *et al.*, 2001), two-component (bvrR-bvrS) regulatory system (Sola-Landa *et al.*, 1998), LPS O chain (Porte *et al.*, 2003) and β-cyclic glucan (Arellano-Reynoso *et al.*, 2005). To survive during the trafficking and in its replicative niche, *Brucella* possesses features to deal with encounter stresses, such as reactive oxygen species (ROS), reactive nitrogen species (RNS), acidic environment, deprivation of nutrients and transient exposure to antimicrobial peptides. For more details on these features, see the following articles (Kohler *et al.*, 2003; Roop *et al.*, 2004).

Despite these escape strategies, some of PRRs finally recognize the pathogen and activate the host innate immune response. Indeed, some studies using the intraperitoneal (i.p.) model have shown a role for TLR9, that recognizes DNA of *Brucella* (Copin *et al.*, 2007; Oliveira *et al.*, 2008). TLR9-deficient mice are more susceptible and show a reduction of IL-12 production by macrophages and DCs and a decrease in IFN_Y and nitric oxide synthase (NOS2/INOS) positive cells in the spleen (Copin *et al.*, 2007; Macedo *et al.*, 2008). A possible collaboration between TLR4 and TLR9 has even been proposed since TLR4-deficient mice are slightly susceptible (Copin *et al.*, 2007). It is recognized that other PRRs recognize *Brucella* because MyD88, which is the adaptor molecule for several TLRs, contributes significantly to the control of *Brucella* infection in mice. Indeed, MyD88-deficient mice are highly susceptible to *Brucella*, maturation of DCs is reduced, IL-12 and TNF_α are less induced and IFN_γ, and INOS production is altered (Macedo *et al.*, 2008; Pei *et al.*, 2012).

To conclude, all the strategies used by *Brucella* impair the ability of the host immunity to rapidly identify *Brucella* as a Gram-negative pathogenic bacterium. The consequence is that classical anti-bacterial host response appears reduced and delayed. It allows *Brucella* to reach its intracellular niche **(Box2)** and establish infection before effective immune activation (de Jong *et al.*, 2010).

3.2. *Brucella* facing the adaptive immune response

Once activated, specialized innate immune cells named Antigen-presenting cells (APCs) (e.g: DCs, Macrophages, B lymphocytes) are able to induce the activation of the adaptive immunity to organize and reinforce the immune defenses against pathogens (for more details on adaptive immunity, see **Box 3** and **Box 4)**. As explained before, *Brucella* limits its detection by the innate immune cells, reduces the maturation of DCs and impairs the antigen presentation to the adaptive immunity to ensure its establishment, but the innate immune cells finally manage to activate lymphocytes that initiate a T helper immune response. The use of RAG1-deficient mice that do not display mature B and T lymphocytes has shown that the adaptive immune response plays an important role in the control of the infection by *Brucella* (Copin *et al.*, 2012; Izadjoo *et al.*, 2000; Vitry *et al.*, 2012).

Box 3: Adaptive immune populations

Adaptive immunity is composed of T and B lymphocytes and can reinforce the immune defenses initiated by innate immunity. Lymphocytes have the ability to recognize a wide range of molecular structures with specific receptors because during their maturation, a process of DNA recombination gives rise to random formation of a large panel of antigenic receptors. Then, selections are necessary to eliminate lymphocytes that recognize self-antigens. For T lymphocytes, the recognition of self-MHC molecules is also an essential selection.

Once mature, naive T lymphocytes generally need to recognize an antigen presented by APCs (e.g.: DCs, macrophages) or infected cells, engage their co-receptor and receive soluble signals (such as cytokines) to be activated. Then, they undergo a clonal amplification before participating in the control of the infection. It is mainly because of this amplification that lymphocytes constitute the second line of defense against a pathogen. T lymphocytes can have a cytotoxic effect by killing the infected cells (e.g.: T $CD8^+$ cells) or produce cytokines that activate other cells to destroy or neutralize the pathogen (e.g.: T helper $CD4^+$ cells). Some of them will differentiate to ensure an immunological memory that provides a long-lasting protection against further contact with the pathogen.

Naive B cells require two distinct signals to be activated: the first one is the recognition of an antigen thanks to their receptors (BCRs). The second signal can be T-cell dependent or independent. The activation of some B cells needs interaction with T cells: in this case, B cells act as APCs: the recognized antigen by BCR is internalized by endocytosis, digested and associated with MHCII on B cell surface. The presentation of the antigen with MHCII triggers the activation of T cells that then give the second signal to the B cells thanks to a variety of cytokines. Alternatively, B cells can directly receive the second signal by certain type of antigens, such as LPS or flagellin (T cell independent activation). Once activated, B cells proliferate and form germinal centers in secondary lymphoid organs where they differentiate into plasma cells or memory B cells. Plasma cells produce antibodies that are particularly effective to fight extracellular pathogens into the blood and the lymph. Globally, antibodies bind foreign antigens that become inactive and/or are better recognized by phagocytic cells for destruction (Bellanti, 2012). The table opposite summarizes the characteristics of the different classes of immunoglobulins (modified from Mesquita Júnior *et al.*, 2010).

Class	Structure	Properties
IgA	Dimeric and monomeric	Found in gastrointestinal, respiratory and urogenital tract mucosa. Prevents the colonization by pathogens. Also present in saliva, tears and milk.
IgD	Monomeric	Membran immunoglobulin. It is part of the membrane receptor of naïve B lymphocytes (BCR).
IgE	Monomeric	Involved in allergic and parasitic processes. Its interaction with basophils and mastocytes causes histamine release.
IgG	Monomeric	Main immunoglobulin of acquired immunity. It has the capacity to cross the placental barrier in certain animals such as carnivores, rodents and primates.
IgM	Monomeric and Pentameric	It is part of the membrane receptor of naïve B lymphocytes (BCR). Form found in the serum, secreted early in acquired immune response.

3.2.1. *Brucella* and T lymphocytes

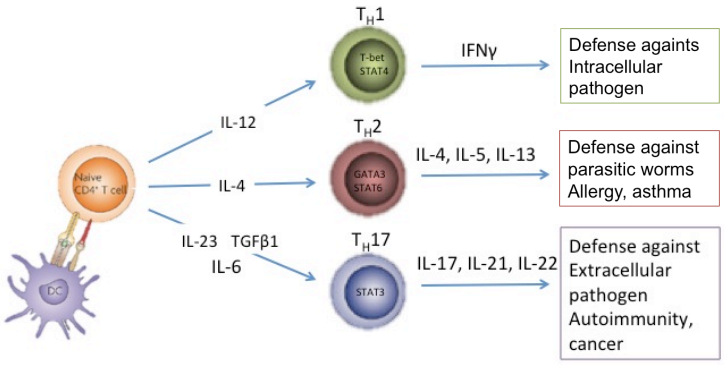
3.2.1.1. Conventional T cell populations

Several research groups have investigated the role of the different T lymphocytes subsets in the defense against *Brucella*. IFNγ is often linked to the control of the infection because its production is crucial to ensure the survival of infected mice (Baldwin & Parent, 2002; Murphy *et al.*, 2001). Moreover, transfer of T lymphocytes has a protective effect partially dependent on IFNγ because an inhibition of the cytokine by antibodies reduces the resistance of the receiver mice (Zhan & Cheers, 1993). Therefore, the protective role of lymphocytes have been associated to their ability to produce IFNγ.

In the ip. model, it is known that $CD4^+$ and $CD8^+$ T cells produce IFNγ during a *Brucella* infection (Baldwin & Goenka, 2006; Copin *et al.*, 2007). However, $CD4^+$ T lymphocytes are the main IFNγ-producers after 10 days of infection by *Brucella melitensis* (Copin *et al.*, 2007). The comparison between Tap1-deficient mice, that do not have mature $CD8^+$ T cells, and MHCII-deficient mice, that do not have mature $CD4^+$ T cells, showed that IFNγ-producing $CD8^+$ T cells are not sufficient to control *Brucella;* IFNγ must be produced by $CD4^+$ T cells for a late control of *Brucella* infection (Goenka *et al.*, 2011; Vitry *et al.*, 2012). The cytotoxic function of CD8+ T cells is neither effective against the bacterium probably because the *B. melitensis* derived protein TcpB inhibits specific activity of these cells against infected cells (Durward *et al.*, 2012). The role of $CD4^+$ T cells against intracellular bacteria is not limited to their ability to produce IFNγ and regulate a Th1 immune response; Indeed, it is known that Th17 can play a major role against intracellular pathogens (Bai *et al.*, 2009; Conti *et al.*, 2009; Tsai *et al.*, 2013). The research team of Muraille and Letesson has used $IL17R_\alpha$- IL21R- and IL22-deficient mice to check the function of Th17 in the protection against *Brucella* infection. The group concluded to a minor role of Th17 because they only observed a slightly higher CFU count in the spleen of these deficient mice 12 days post-infection (Vitry *et al.*, 2012). Recently, a cytotoxic function of $CD4^+$ T cells has been identified in a mouse model of *B. abortus* infection. If early stage of the infection is characterised by a Th1 adaptive immune response with the production of IFNγ, it seems that an increasing percentage of $CD4^+$ T cells also produce granzymes B and have a cytolytic activity against infected phagocytes by *B. abortus.* So far, little is known about the phenotype, function and transcriptional profile of cytotoxic $CD4^+$ T cells (Martirosyan *et al.*, 2013).

Box 4: T cell immune responses

T CD4$^+$ lymphocytes activated by APCs with MHCII molecules can induce three major well-described sub-classes of immune responses, depending on the type of detected pathogen: Th1, Th2 and Th17 immune response (Kaiko *et al.*, 2008). Globally, Th1 is adapted to fight intracellular pathogens, Th2 limits the invasion by extracellular macro-parasites such as helminthes (Mosmann & Coffman, 1989), and Th17 is generally observed as a response against extracellular bacteria (Harrington *et al.*, 2006)(Figure opposite). It is however important to note that there are exceptions of these generalities, depending on the situation and location of the infection. It has been reported, for example, that Th17 confers partial protection against intracellular bacteria such as *Mycobacterium tuberculosis* in lungs (Okamoto Yoshida *et al.*, 2010). These last years, the knowledge on immune responses has evolved and new T helper subsets of lymphocytes have been discovered but still have to be investigated: Th9 lymphocytes produce IL-9 and seem to play a role in inflammatory diseases such as asthma (Kaplan, 2013), and T helper follicular lymphocytes (Thf) are important for the establishment of germinal centers and the differentiation of B lymphocytes into plasma cells and memory B cells (Crotty, 2011).



APCs and non-immune infected cells can also activate T CD8$^+$ lymphocytes by the presentation of antigens with MHCI molecules. It is generally the case when the pathogen is mainly located in intra-cytoplasmic compartment. Activated T CD8$^+$ cells have a cytotoxic effect on the infected cells: they induce apoptosis in target cells by the release of lytic granules containing effector proteins. Perforins and granzymes are the two main types of cytotoxic proteins that induce the death of the target cells (**Box1**) (Murphy, 2011).

Other T cell types can take part in the immune response. Natural Killer (NK) T cells are activated by one particular PAMP (glycolipid) presented with a complex structurally close to MHCI, named CD1. NKT cells produce a large panel of cytokines, such as IFNγ, IL-4 and IL-13, few minutes after antigenic stimulation that rapidly modulate the immune response (Godfrey *et al.*, 2010). γδT cells are generally compared to innate immune cells because they are directely activated by non-peptidic antigens and are present in cutaneous epithelium and mucosal surfaces. They are an important source of inflammatory cytokines such as IFNγ and IL-17 (Roark *et al.*, 2008). These cells can also ensure an immunological memory (Sheridan *et al.*, 2013).

After immune activation, a particular type of T CD4+ cells, named regulatory T (reg) cells, is able to ensure the end of the stimulatory activities of T cells to suppress potentially deleterious effects that would generate immunopathology. They produce regulatory cytokines, such as IL-10 and transforming growth factor (TGF)-β, to feedback control the pro-inflammatory immune response (Corthay, 2009; Durward *et al.*, 2012; Noora Ottman, 2012)

3.2.1.2. Non conventional T cell populations

γδ T lymphocytes : The lymphoid tissues of mice contains a small number of γδ T lymphocytes. These cells are mainly abundant in barrier surfaces such as lungs, intestine and skin, and play an important role against infection of intracellular bacteria, such as *Mycobacterium tuberculosis* (Janis *et al.*, 1989; McGill *et al.*, 2014), *Listeria monocytogenes* (Tramonti *et al.*, 2008) and *Salmonella choleraesuis* (Emoto, 1992). It has also been proposed that these non conventional lymphocytes could alter the response against Gram-negative bacteria such as *Klebsiella pneumoniae* (Moore *et al.*, 2000). The role of γδ T lymphocytes in brucellosis has not been deeply investigated. It is known that these cells are activated and proliferate in the blood of humans infected with *Brucella melitensis* (Bertotto *et al.*, 1993). Human γδ T cells (Vc9Vd2 T cells) activated by a "fraction" of *B. suis* produce TNFα and IFNγ, which reduces bacterial multiplication inside infected cells (Ottones *et al.*, 2000). It has been demonstrated that bovine γδ T cell rapidly respond to *Brucella abortus* infection of co-cultured autologous macrophages and could impair the intracellular replication of the pathogen thanks to the production of IFNγ (Skyberg *et al.*, 2011). Experiments with intra-peritoneal infection of mice with *Brucella abortus* have demonstrated that γδ T cell-deficient mice are slightly more susceptible than wild-type (wt) mice during the first week of infection. It is translated into a splenomegaly and a higher bacterial load in the spleen. These cells produce IFNγ and IL17, and their ability to confer protection seems also to be linked to the production of TNFα by splenocytes. However, their role seems to be limited because the bacterial load decreases rapidly to similar levels as the wt mice (Skyberg *et al.*, 2012).

NK T lymphocytes : The role of these cells during an infection of *Brucella suis* has been investigated *in vitro* by the research team of Lafont. One population of NKT cells, the $CD4^+$ invariant NKT cells, limit the growth of *Brucella* in macrophages thanks to their cytotoxic function. They trigger apoptosis of infected cells by a FAS-FASL interaction and by the release of lytic granules (Bessoles *et al.*, 2009). *In vivo* investigations would be necessary to confirm a potential role of these cells.

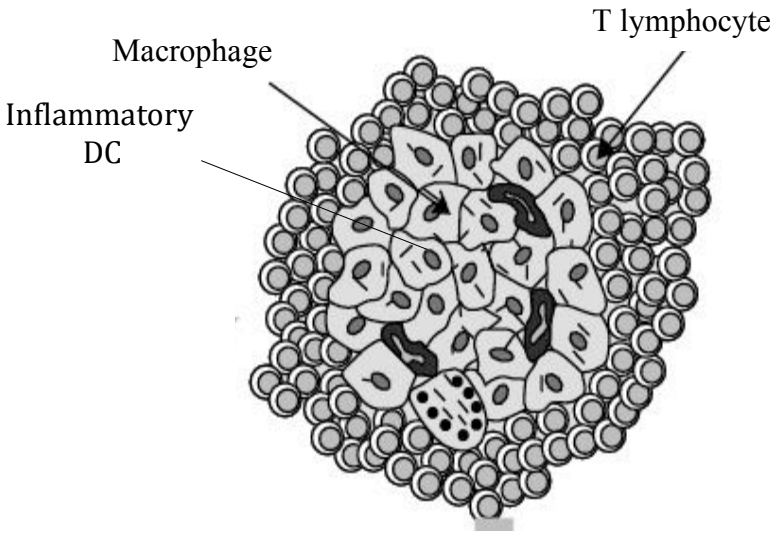


Figure 3: Schematic structure of a granuloma (source: adapted from Jacobs M., 2007)

3.2.2. Brucella and B lymphocytes

It is now agreed that B-lymphocytes do not contribute to the control of primary infection by *Brucella*. On the contrary, in the i.p. model of infection, B cell-deficient mice are better at clearing the bacteria from the spleen thanks to the absence of the regulatory function of these cells. B-lymphocyte production of TGF-β in BALB/c mice and IL-10 in C57BL/6 mice at the early stage of the contamination dampens the pro-inflammatory Th1 response and thus improves the ability of *Brucella* to establish the infection (Goenka *et al.*, 2011; Vitry *et al.*, 2012). Moreover, Baldwin's research team has demonstrated that B-lymphocytes can provide an intracellular niche for *Brucella* and could participate in the chronic infection (Goenka *et al.*, 2012).

3.3. Granuloma: a strategy of the immune system against *Brucella*

Granulomas are organized dynamic structures mainly composed of activated monocytes surrounded by T lymphocytes **(figure 3)**. Granuloma formation is generally presented as a fighting mechanism against a pathogen and its dissemination. But it has also been observed that this host strategy can be exploited by the pathogen to ensure its expansion and dissemination (Davis & Ramakrishnan, 2009; Ottones *et al.*, 2000; Ramakrishnan, 2012). Granuloma and their contradictory role in the disease are often described for chronic infection induced by *Mycobacterium tuberculosis* (Co *et al.*, 2004). Our group has described the formation of granulomas in spleen of mice intraperitoneally infected with *Brucella melitensis* (Copin *et al.*, 2012) (see appendix 1). One week post-infection granulomas are located in red pulp of the spleen and are composed of mature activated monocytes, surrounded by T lymphocytes and granulocytes. After twelve days, their phenotype is similar but we observe a re-localization of granulomas into the white pulp (Copin *et al.*, 2012). During brucellosis, their formation seems to be necessary to control the bacterial load and it seems to be dependent on the Th1 immune response since MyD88-, IL12- and IFNγ-deficient mice display altered granuloma structure and are highly susceptible to the infection (Copin *et al.*, 2012).

3.4. Secondary infection and immunity

When *Brucella* infects an animal, immune mechanisms of the host are set up to fight the pathogen and some immune cells differentiate to ensure an immunological memory that provides a long-lasting protection against further contact with the pathogen.

Most of the data known on immune memory against *Brucella* come from the I.P. model of infection. If humoral immunity does not positively contribute to the control of a primary infection (Goenka *et al.*, 2011; Vitry *et al.*, 2012), our group has shown that humoral defense is essential to limit early blood dissemination of a challenge (second infection). This step seems to be important to ensure clearance of the spleen, as B cell-deficient (MuMT) mice are not able to avoid bacteria persistence in the spleen in comparison with wild-type mice. AID-deficient mice that only produce IgM isotype of antibodies are as resistant as the wt mice; it suggests that IgM are sufficient to guarantee the protection. It is important to highlight that injection of live as well as heat-killed bacteria induces the development of B-cell memory population able to produce specific circulating IgM fighting against a second bacterial dissemination (Vitry *et al.*, 2014a) (see appendix 2).

Cellular immunity also plays a key role into the fighting of a secondary infection. Our group has shown that, as for the control of a primary infection, IFNγ-producing $CD4^+$ T cells are crucial for the control of a challenge. More precisely, $CD4^+$ T cells are not important to control early blood dissemination but later to eradicate *Brucella* from the spleen. It was proven by the use of mice deficient for activated $CD4^+$ T cells and IFNγ-inducing pathways: MHCII-, MyD88- and IL-12-deficient mice, associated with a drastic reduction of IFNγ, have serious impairment of protective immunity in the spleen. It is also essential to point out that cellular protection is only ensured following live vaccine administration. Heat killed *Brucella* does not induce the development of CD4+ lymphocyte memory population able to rapidly produce IFNγ in case of re-infection. It means that the dynamics of *Brucella*'s intracellular cycle is critical to induce this effector mechanism (Vitry *et al.*, 2014a) (see appendix 2).

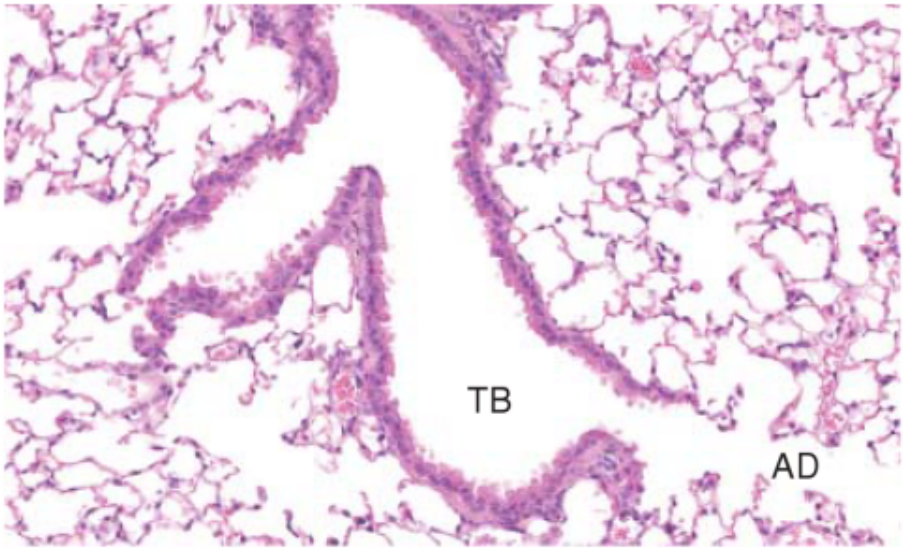


Figure 4: Histology of a lung section in mouse. Terminal bronchiols (TB) lead to alveolar ducts (AD). (source: C.L. Scudamore 2014)

4. Mucosal environment, first interface with microorganisms

4.1. Overview

The mucosal surfaces of the respiratory, gastrointestinal and urogenital tracts are in direct contact with a large collection of foreign antigens from the external environment. These tissues must maintain a barrier to avoid infection and ensure survival of the host because they are the preferred entry routes for most of the pathogens. In parallel, these surfaces have to preserve a peaceful relationship with diverse beneficial microbial communities (Noora Ottman, 2012) (Ichinohe *et al.*, 2011) and be tolerant to a large panel of innocuous environmental stimuli. Therefore, the challenge of mucosal surfaces is to protect the body against invading pathogens while maintaining symbiosis with commensal microbiota and not responding to innocuous antigen (Belkaid & Artis, 2013).

4.2. Respiratory tract

4.2.1. Anatomy and functions

The respiratory tract can be divided into two main parts: (i) the upper respiratory tract, composed of the nasal cavity, the mouth and the nasopharynx, mainly involved in the filtration, humidification and temperature's adjustment of the inhaled air and (ii) the lower respiratory tract, also named the broncho-pulmonary apparatus, which is responsible for the conduction of the air and the gaseous exchanges. The latter begins by the larynx and continues with the trachea that is divided into two primary bronchi. They are then divided in secondary and tertiary bronchi before splitting into many small ducts, the bronchioles. Terminal bronchioles mark the end of the conducting airway and lead to the alveolar ducts, increasingly implicated into gas exchange (**Figure 4**). They end in dilated spaces, named the alveolar sacs, opening with alveoli (Wheather et al., 2001; Scudamore, 2014)

The respiratory tract is drained by cervical, mediastinal and tracheobronchial lymph nodes (LNs) (Gueirard *et al.*; Van Den Broeck, 2006). They are secondary lymphoid organs containing B cells, T cells and other immune cells such as DCs and macrophages, and act as filters by trapping aberrant cells and pathogens traveling through the lymph. It is a site where T lymphocytes are activated by APCs before their migration to the infected site (Katakai *et al*., 2004)

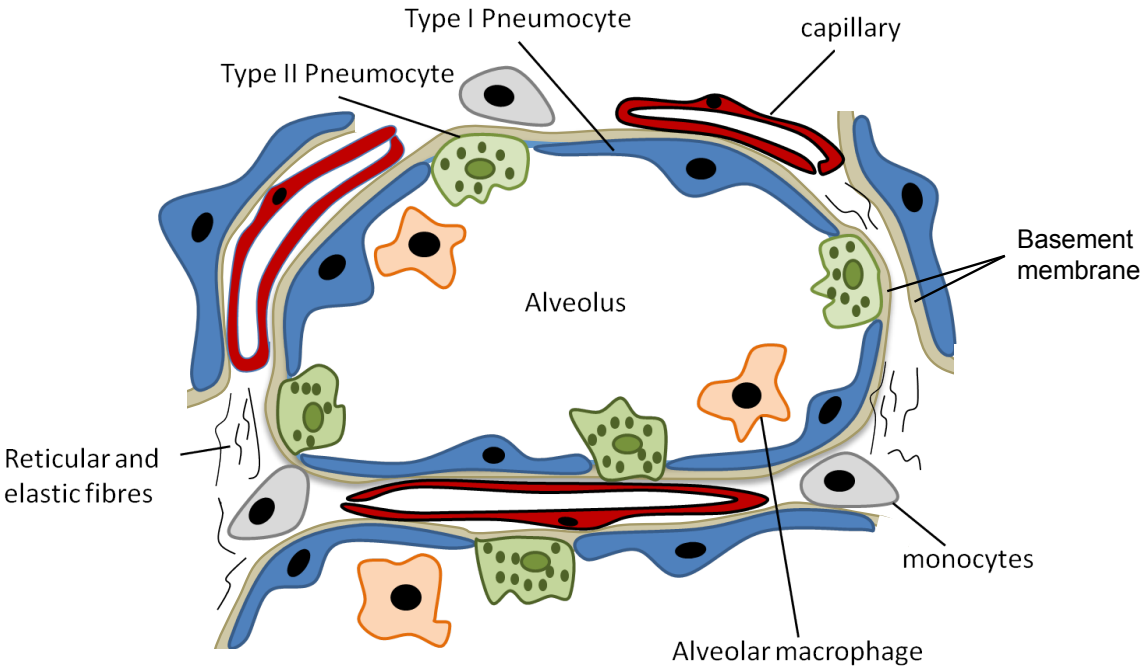


Figure 5: Schematic representation of the main constituents of an alveolus and the interalveolar wall. (Source: adapted from the histology guide, University of Leeds)

4.2.2. Histology of the mucous membrane

The upper respiratory tract is covered by a pseudostratified cylindrical epithelium and many caliciform cells that secrete mucus. The epithelium is rested on a loose layer of collagen, named chorion or lamina propria. The epithelium and the chorion constitute the airway mucosa (Conti *et al.*, 2004; Wheater *et al.*, 2001).

The epithelium of the airway progressively evolves from a ciliated pseudostratified cylindric type in the larynx and trachea, to a non ciliated simple cubic type in the distal airways. There are a lot of caliciform cells in the trachea and their number decreases progressively so that they are absent in the terminal bronchioles (Conti *et al.*, 2004). Endocrinian cells are scattered among the epithelium and are responsible for secretion of mediators such as serotonin, calcitonin and bombesin. Immune cells, such as DCs and macrophages, are particularly abundant into the mucosa and ensure the immune-surveillance within and beneath the surface of the epithelium. $CD8^+$ T cells, that have a cytotoxic effect on infected cells, are mainly located among epithelial cells while $CD4^+$ T lymphocytes, which ensure development of the proper immune response to fight the entering pathogen, are preferentially located in the lamina propria (Harbeck, 1998). We can also find clusters of immune cells, part of the mucosa-associated lymphoid tissue (MALT), named BALT for «Bronchus-Associated Lymphoid Tissue ». Typically, it is a place where antigen sampling occurs and immune responses are initiated. It seems that in human and mice, BALT develop mainly in response to microbial stimulation or inflammation. They are located throughout the lung, usually between bronchi and pulmonary arteries. The center is composed of a B follicule and a T zone, and DCs and macrophages are scattered throughout the BALT (Cesta, 2006; Randall, 2010). Finally, deeper in the mucous membrane we can find a smooth muscle layer that increases progressively to be maximal in the distal airways. It is important to control the air flow all along the tract (Wheater *et al.*, 2001). If we focus on the alveoli and their environment (**figure 5**), they are composed of two cell types : type I pneumocytes, which are flat cells that consitute most of the surface of the alveoli, and type II pneumocytes, which are cells that produce the surfactant[1]. Each alveolus is surrounded by

[1] Surfactant : a complex of lipoproteins that increases the ability of lung to expand and prevent the collapse of the lung (Goerke, 1998).

a network of capillaries, and type I pneumocytes are in close contact with endothelial cells for the gaseous exchanges. The wall between alveoli can be extremely thin to let the air through, or thicker and display conjunctive tissue with collagen, elastic fibers or fibroblast. This interstitial space is also endowed with few interstitial macrophages, DCs and T cells. Alveolar macrophages, also called « dust cells » are located in alveolar space and capture foreign particles reaching the alveoli. They are coming into the lungs from blood and differentiate into effective cleaner (Gartner & Hiatt, 2004).

4.2.3. Immunity of the broncho-pulmonary apparatus

4.2.3.1. In steady state

Almost two-thirds of microbial agents enter the body through the respiratory tract (Bellanti, 2012). It is then easy to understand that mammals have developed an immune system specifically dedicated to this particular surface. In steady state, a combination of mechanisms limits inflammatory reaction to frequent innocuous environmental antigens and allows the symbiotic relation with favorable commensal bacteria:

- Mucus produced by caliciform cells and the submucosal glands traps foreign particles that enter into the airways during respiration, and contains antimicrobial peptides secreted by epithelial cells and immune cells, such as defensins or cathelicidins, that kill bacteria by compromising cell wall integrity or inhibit their growth. The cilia of the epithelial cells beat to move the secreted mucus toward the laryngopharynx where it will be expectorated or swallowed to the stomach (Beisswenger et al., 2005; Lillehoj et al., 2002).
- Another mechanism involves dimeric immunoglobulin A (IgA). They are produced as monomers by plasma cells from the lamina propria, they are dimerized during their transcytosis across the epithelium and are secreted from the apical surface of epithelial cells. They guard the epithelium from microbial entry, and transport antigens or microorganisms that penetrate the barrier back into the lumen (Nagler-Anderson, 2001).
- Immune receptors of the mucosal membrane seem to be down regulated or modified to avoid an inappropriate immune response against non-pathogenic bacteria. For example, epithelial cells are reported to be weakly responsive to TLR2-

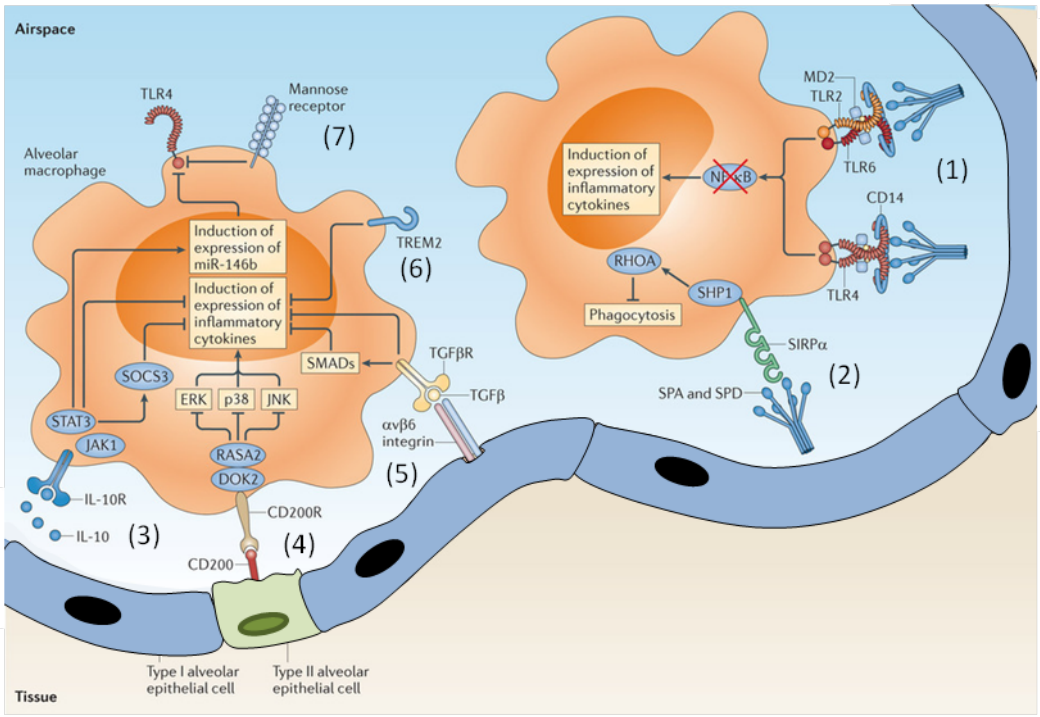


Figure 6: Negative regulation of alveolar macrophages by soluble mediators and by cell–cell interactions with bronchial and alveolar epithelial cells. **(1)** Pulmonary surfactant-associated protein A (SPA) and SPD are abundant in the airways and block TLR2 and TLR4 interactions with their ligands, as well as with the co-receptors MD2 and CD14, thereby stopping the initiation of the inflammatory response through the activation of nuclear factor-κB (NF-κB). **(2)** Attachment of SPA and SPD to signal-regulatory protein-α (SIRPα) recruits SH2 domain-containing protein tyrosine phosphatase 1 (SHP1) and activates RHOA, which inhibits phagocytosis. **(3)** Interleukin-10 (IL-10) is abundant in lung and induces the expression of negative regulators such as suppressor of cytokine signaling 3 (SOCS3) and the microRNA miR-146b through Janus kinase 1 (JAK1)–signal transducer and activator of transcription 3 (STAT3) pathway. SOCS3 inhibits the expression of pro-inflammatory cytokines. **(4)** The CD200 receptor (CD200R) interacts with CD200 on the respiratory epithelium. It blocks the extracellular signal-regulated kinase (ERK), p38 mitogen-activated protein kinase (MAPK) and JUN N-terminal kinase (JNK) inflammatory pathways through recruitment of docking protein 2 (DOK2) and RAS GTPase-activating protein RASA2. **(5)** The αvβ6 integrin, expressed on bronchial and alveolar epithelial cells, binds transforming growth factor-β (TGFβ), and facilitates access of the TGFβ receptor (TGFβR) on alveolar macrophages. It controls inflammation through both SMAD-dependent and SMAD-independent signaling pathways. **(6)** Triggering receptor expressed by myeloid cells 2 (TREM2) limits inflammation in macrophages by an unknown mechanism. **(7)** The mannose receptor (CD206) blocks TLR4 and inhibits phagocytosis of pathogens. (Source: adapted from Hussel *et al.*, 2014)

- dependent ligands of gram-positive bacteria; it correlates with a low expression level of TLR2 and missing expression of co-receptor CD36 (Mayer *et al.*, 2007). Another example is TLR4 that seems to be only expressed in the intracellular compartment of epithelial cells to avoid an inappropriate activation due to frequent exposure to air containing small amounts of LPS (Guillot *et al.*, 2004).
- Resident alveolar macrophages are negatively regulated by many soluble mediators and interaction with epithelial cells (**figure 6**). Thus, in steady state, their phagocytic activity is used to sequester the antigens reaching the alveoli to protect local tissues from the development of specific immune response (Hussell & Bell, 2014; MacLean *et al.*, 1996).
- There are different subsets of resident lung DCs the role of which depends on the location and the context of the lung environment but it seems that during the steady state, DCs have an "immature" status where they protect the lung from inflammation and damages (Plantinga *et al.*, 2010; Kushwah et al., 2011).
- By default, the immune response in lungs is non-inflammatory; T-cell mediated tolerance (Holt, 1994). The mechanisms ensuring this "default" immune response is not well understood but it seems that immature DCs participate to the tolerance by the induction of regulatory T (Treg) cell populations (Kushwah *et al.,* 2011). Treg then regulate T-cell responses thanks to mechanisms such as the production of IL-10 and TGF-β (Strickland *et al.*, 2008).

4.2.3.1. During infection

In case of imbalance in the system like after tissue injury induced by physical agents or because of impairment of the immune system, some commensals can overgrow and invade the tissues; they become pathogens and are named pathobionts. Other bacteria are non-commensal but need a host for their life cycle and display mechanisms to get into the tissues despite physical and immunological barriers. Directly after invasion, PAMPs are recognized by PRRs, expressed on epithelial cells, sentinel alveolar macrophages and DCs. The latter migrate to the draining lymph nodes to activate naive T cells and initiate the immune response. Despite the fact that alveolar macrophages are able to migrate to the draining lymph node (Kirby *et al.*, 2009), they do not seem to have a significant role of APC (Hussell & Bell, 2014). But they can be activated by stimuli, such as cytokines or microorganisms. They are detached from airways epithelial cells and the action of regulatory ligands is canceled. In

this activated condition, macrophages phagocyte, kill, produce cytokines and coordinate the innate immune response (Holt *et al.*, 2008; Hussell & Bell, 2014).

Effector "Th" responses are extremely complex and heterogeneous. However, we can highlight the importance of Th17 cells at mucosal surfaces, and particularly in the lungs. It has been demonstrated that Th17 cytokines, such as IL-17A, play a key role for effective host defense against extracellular or intracellular pulmonary pathogens (Tsai *et al.*, 2013; Way *et al.*, 2013). Interestingly, Th17 and Treg cells seem to be mutually exclusive. In mice, TGF-β induces Th17 and Treg but TLR activation also induces IL-6 and the resultant is the action of Th17 cells. IL-21 also participates in the differentiation of Th17 cells that produce IL-17, IL-22 and IL-23. This response ensures the recruitment of neutrophils and cytotoxic molecules to the lungs. Neutrophils produce ROS, proteolytic enzymes and other antimicrobial peptides to kill foreign pathogens in the infection site (Way *et al.*, 2013). $CD4^+$T lymphocytes, producing Th17 cytokines, are essential in the adaptive phase of the host defense. However, innate immune cells, such as γδ T cells, NK cells and NKT cells, are able to produce IL-17A earlier in the lungs and make the link between the innate and adaptive immune response (Way *et al.*, 2013). For example, γδ T cells are the major source of early production of IL-17A in response to infections like *K. pneumonia* (Price *et al.*, 2012), *M. tuberculosis* (Peng *et al.*, 2008) or *M. bovis* (Okamoto Yoshida *et al.*, 2010). Rapid production of IL-17A can even promote the adaptive immune response Th1 into the lungs to improve pathogen clearance (Umemura *et al.*, 2007; scurlock *et al.*, 2011). In some cases, however, the function of Th17 can become pathogenic and IL-17A have deleterious effect when the production of neutrophils does not improve the clearance of the pathogen, leading to a prolonged inflammation and damage in the tissue generating pathologies such as cystic fibrosis (Way *et al.*, 2013; Jin *et al*., 2013).

4.2.4. Infection with inhaled *Brucella*… what's going on?

Intranasal or aerosol infection by *Brucella melitensis* has not been deeply investigated. Intranasal infection by *Brucella* leads to the infection of lungs followed by progressive dissemination into the spleen and the liver. In the i.p. model, however, the dissemination is immediately systemic. It highlights the fact that the way of infection could have significant impact on the immune response. The research team of Gorvel has shown that alveolar macrophages are the first infected cells in the lungs, and that *Brucella* survives and replicates

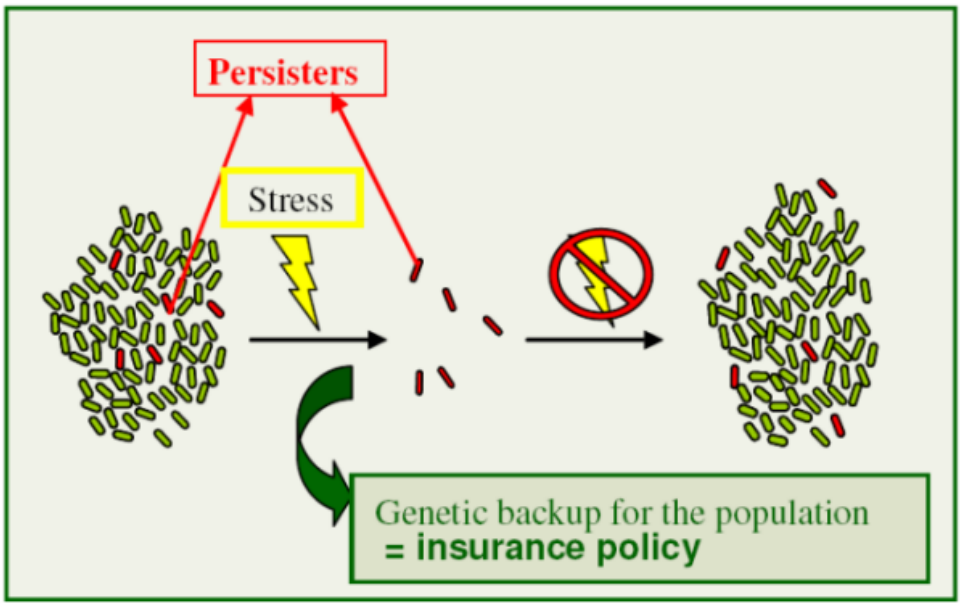


Figure 7: Schematic representation of persister cells. When the bacterial population undergoes a lethal stress, such as immune defense or antibiotic treatment, a fraction of the cells survives. After the stress, the cells give birth to a similar population than initial one. (source: http://www.biw.kuleuven.be/dtp/cmpg/spi/persistence.aspx)

for 2 to 5 days after intranasal infection (Archambaud *et al.*, 2010). A recent study made with an aerosol infection has shown that TLR2 and TLR4 play a role to clear lung infection but do not have significant contribution in spleen and liver (Pei *et al.*, 2012). The research team of Hoover did not find any gross or microscopic indication of inflammation all along the infection in the lungs, in opposition to what it is happening in deep organs such as the spleen and liver where recruitments and granulomas are detected (Mense *et al.*, 2001). Another group has shown the importance of T and B lymphocytes thanks to Rag1-deficient mice that do not control the infection and have persistence in lungs, whereas wt mice progressively eliminate the bacteria from this organ (Izadjoo *et al.*, 2000). *Brucella* is progressively eliminated from the lungs but it is able to reach other organs like the spleen where it persists for a long time. The next chapter will discuss the ability of intracellular bacteria to persist in the host and to take advantage of the host cells.

5. Persistence of bacteria in the host

5.1. Overview

Innate and adaptive immunity usually eliminates the acute infection caused by pathobionts or strict pathogens. In some cases, however, bacteria are able to survive in the host. The prolonged presence of bacteria in the host, or persistence, is possible thanks to strategies that can be divided in two categories (i) the invader is adapted to the environment and/or (ii) it must induce a local reconfiguration of the environment.

The adaptation of bacteria to the environment can be achieved by multiple mechanisms. For example, *Neisseria meningitidis*, a human commensal that can be responsible for the acute inflammation meningitis and occasionally chronic infection (stephens, 2009), has a major defense system: a capsule made of homopolymers of α2-8-linked sialic acid. The steric conformation of this sialic acid seems to prevent the antimicrobial peptides to reach the outer membrane (Gasparini *et al.*, 2012) and it is poorly immunogenic because it mimics human neural cell adhesion molecule (Lo *et al.*, 2009). The bacterium is also equipped with other surface structures of which the composition is close to the host's ones. It is the case of its LPS: one epitope, the Lacto-N-neotetraose, is a close copy of a red blood cells antigen. This bacterium also displays an extremely variable genome and is able to acquire genes of other bacteria (horizontal gene transfer) (Tettelin, 2000). This kind of mechanism allows the

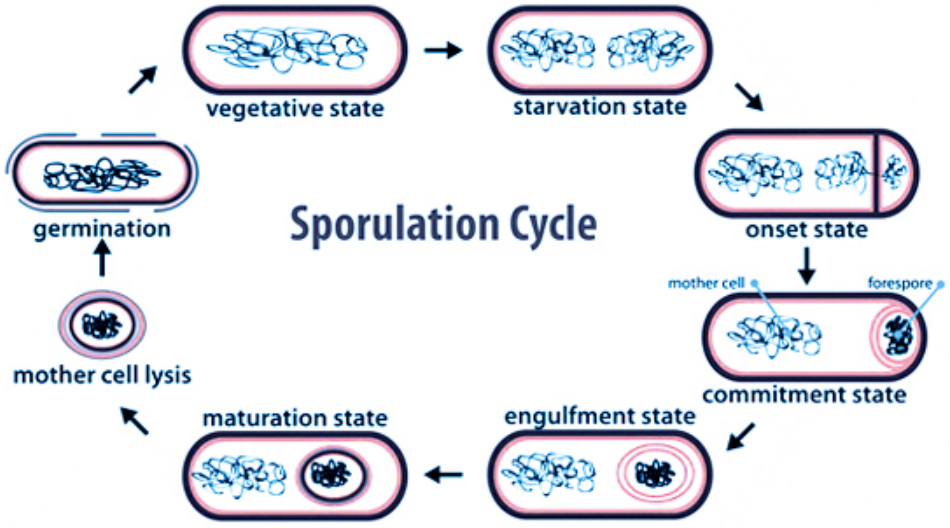


Figure 8: Schematic sporulation cycle under a stress condition, such as starvation, the bacterium (e.g. *Bacillus subtilis*) produces a spore to survive. The spore is then able to give rise to a bacterium (germination) when the stress is ended. (source: http://ezines.tifn.nl/intouch/october2013/eijlander_sporeweb.html).

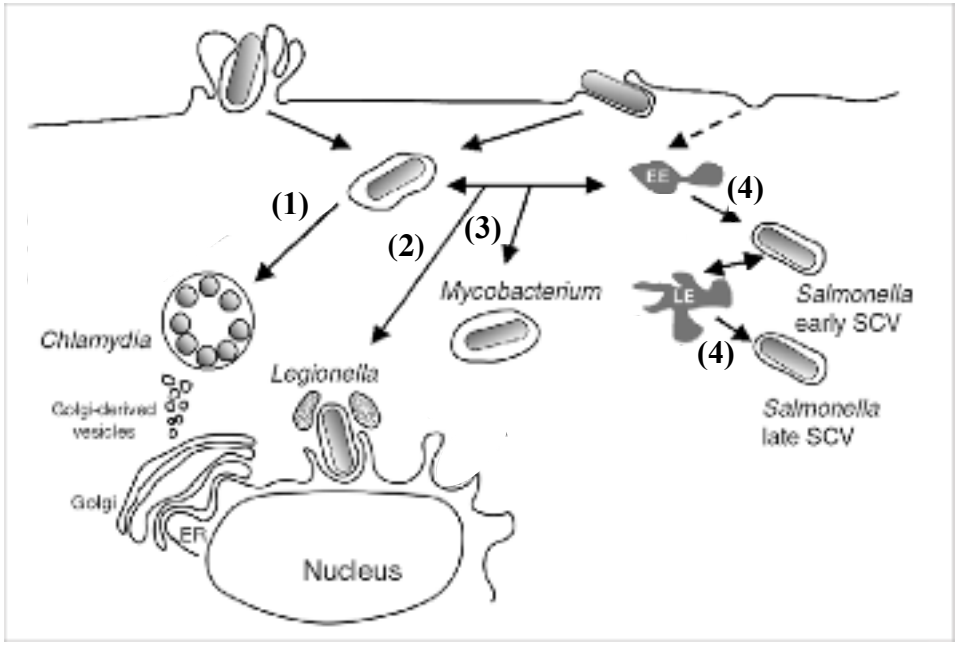


Figure 9: Hijacking of host cell trafficking by pathogens. **(1)** Separation from the endocytic route and interaction with Golgi-derived vesicles to form an inclusion vacuole. **(2)** Segregation from the endocytic route at the early endosome (EE) stage and formation of an endoplasmic retuculum-like compartment. **(3)** Arrest of the maturation at EE stage. **(4)** Transient arrest of the bacterium-containing vacuole at the EE and late endosome (LE) stages. SCV=*Salmonella* containing vacuole. (Source: adapted from A. A. F. Garcia-del Portillo, 2004)

bacterium to avoid microbicidal immune defenses and to establish a preferential niche to replicate. The adaptation can also be achieved by a small number of bacteria, named persister cells, that are dormant variants of regular bacteria formed randomly in the population, as it is the case for *Pseudomonas aeruginosa,* a pathobiont responsible for nosocomial infections (Lewis, 2010) (**Figure 7**). Persister cells are highly tolerant to harmful conditions, allowing long-term surviving in the host. In *Bacillus anthracis*, responsible for anthrax infection, it is dormant spore that can be recovered in lungs months after inhalational infection (Jenkins & Xu, 2013). Spores are produced by bacteria in detrimental conditions and display a hard protective coating that contains key elements of the bacterium. These resistant structures turn into cells back when the conditions become more favorable (**figure 8**).

Bacteria can also display mechanisms that modify the environment to generate a favorable condition for their survival. *Chlamydia, Legionella, Mycobacterium* or *Salmonella*, deviate the host's intracellular trafficking to prevent the fusion of their vacuole with lysosomes and to create a specialized organelle favorable for replication (**Figure 9**). To achieve the deviation of the host trafficking, they translocate effectors across the vacuolar membrane thanks to a secretion system. Then the effectors manipulate host proteins located in the cytoplasm or on cellular organelles (Alix *et al.*, 2011). Another example involves the diarrheagenic enteropathogenic *Escherichia coli*. It limits the host cell death because it is able to interfere with apoptotic pathway of infected enterocytes (intestinal epithelial cells) early after infection. It is also the secretion of at least one effector (EspZ) and its interaction with host proteins that ensure the survival of the cell (Roxas *et al.*, 2012).

5.1. Reservoir cells for pathogens

Various bacterial pathogens have their own strategies to overcome the immune defenses of the host. Intracellular life is a way in itself to avoid a series of defenses such as complement and antibodies. Different cell types are targeted by bacteria to constitute a niche on the long term. Infection of DCs are particularly strategic for bacteria because these cells have many functions in the immune defense. It is well described for *Mycobacterium tuberculosis* that suppress maturation and migration of DCs, causing a delay of the adaptive immune response (Urdahl *et al.*, 2011). *Salmonella enterica* reduces the antigen presentation by DCs and seems to use their migratory function to spread in new

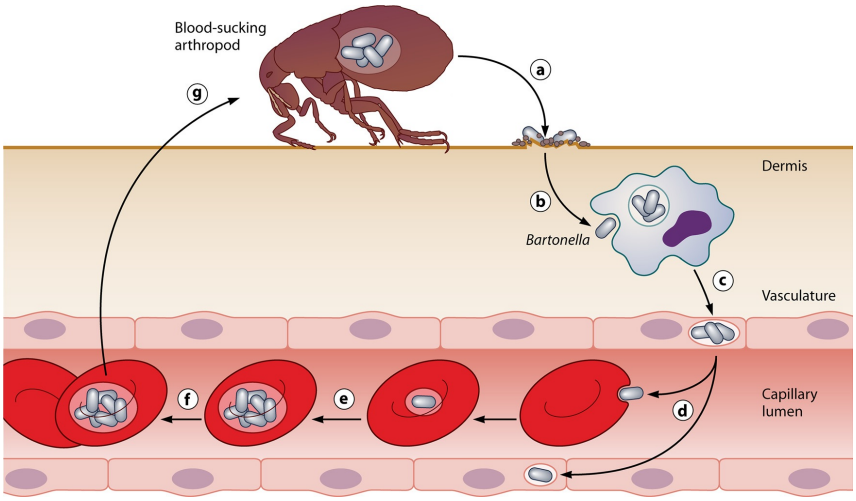
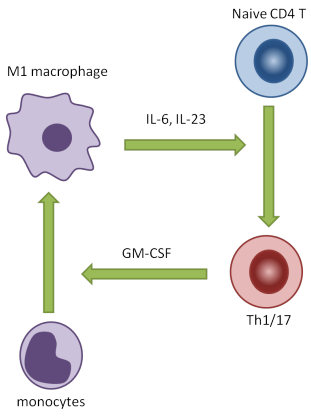


Figure 10: Infection strategy of *Bartonella*. **(a)** Insect vectors transmit the bartonellae to human. **(b)** Bacteria are probably transported by migratory cells before **(c)** reaching vascular endothelium. **(d)** Bartonellae then spread into the bloodstream where they invade erythrocytes, **(e)** replicate and **(f)** persist in the erythrocytes before **(g)** being aspirated by a bloodsucker insect. (source: Harms et al. 2012)

BOX 5 : correlation between T helper immune responses and polarization of macrophages

Scientists have initially discovered that macrophages activated by the Th1 cytokine IFNγ, and LPS, display a phenotype and a metabolism different from macrophages activated by Th2 cytokines (IL-4 and IL-13). Therefore Th1-induced macrophages and Th2-induced macrophages were named M1 (classically activated macrophages) and M2 (alternatively activated macrophages) respectively (Martinez *et al.*, 2014). Subsequently, Th17 immune response has been discovered and it is now admitted that M1 macrophages are not always only associated to Th1 but also to Th17 immune response. Inflammatory M1 macrophages can produce TNFα, IL-6 and IL-12 that promote the expansion and the activation of IFNγ-producing $CD4^+$ cells (Th1); but these macrophages can also produce IL-23, which is an important cytokine that acts through its receptor, IL-23R, expressed on $CD4^+$ T cells, to promote the development of IL-17-producing cells (Th17) (Krausgruber *et al.*, 2011). It has also been demonstrated that IL-23 can give rise to an $IL\text{-}17^+IFN\gamma^+$ population of T cells. This phenomenon supports the idea of a close relationship between Th1 and Th17 cells. M1-induced Th1/17 cells can produce GM-CSF that facilitates the differentiation of M1 macrophages. Opposite figure shows this positive feedback loop, generally associated with inflammation in autoimmune diseases (Li et al., 2013).



However, it is important to mention that Th17 immune response is not always associated with M1 macrophages: for example, mycobacterial-infection-induced suppressor macrophages (MIS-MΦ) down regulate T cell production of Th1 and Th2 cytokines but up-regulate Th17 cells expansion that increase production of IL-17A and IL-22 cytokines. This population of macrophages express markers of M1 such as IL-12 or iNOS but they also display M2 markers, such as IL-10 or Ym1. This phenotype is distinguishable from Th1-induced M1 and Th2 -induced M2 macrophages subsets (Tatano et al., 2014). These observationes reinforce the concept of plasticity of macrophages described in the point 5.2.1.

environment and particularly to mesenteric lymph nodes (MLNs) (Cheminay *et al.*, 2005). The uropathogenic *Escherichia coli*, responsible for the urinary tract infection, is able to get into the superficial epithelial cells of the bladder thanks to a filamentous adhesive organelle named Type 1 pili. Once in the cell, it persists and can multiply before invading neighboring cells (Martinez, 2000). *Bartonella baciliformis* and *B. quitana,* causing human disease transmitted by insect vectors, have a tropism for erythrocytes. Actually, it seems that from the dermis penetration, they get into a first niche, probably including vascular endothelium, before spreading in the red blood cells where they cause a long lasting intra-erythrocytic bacteraemia (Eicher & Dehio, 2012) (**Figure 10**). Macrophages are another cell type regularly targeted by intracellular bacteria, such as *Francisella tularensis*, *Salmonella sp.* or *Mycobacterium tuberculosis*. The next point describes these cells and the reasons why they are particularly appreciated by pathogens.

5.2. Focus on macrophages

5.2.1. Macrophage plasticity

Macrophages are innate immune cells that perform a large panel of functions, in health but also in response to infection. Phagocytosis, to kill pathogens or eliminate tissue debris and apoptotic cells, was the first function discovered by Elie Metchnikoff at the beginning of the 20th century (Martinez *et al.*, 2009). It was later admitted that their functions are much more diversified. Indeed macrophages also make the link between the innate and the adaptive immunity by activities such as presentation of antigens and secretion of cytokines, and they take part in the repairing of wounded tissue and tissue remodeling (Shapiro *et al.*, 2011). The ability of resident and recruited macrophages to perform distinct functions depends on environmental signals, such as cytokines, pathogen derived molecules and lipid mediators (Kiss *et al.*, 2013). These stimuli give rise to macrophages with a large variety of phenotypes and different functional states.

Since the discovery of macrophage diversity, macrophages have been classified in two groups: classically (M1) and alternatively activated (M2) macrophages (Mantovani *et al.*, 2004). This division follows the lymphocyte terminology: Th1 and Th2 subsets (to have more information about the correlation between T helper immune responses and the polarization of macrophages, see **Box 5**). The M1 phenotype is generated during cell-mediated immunity

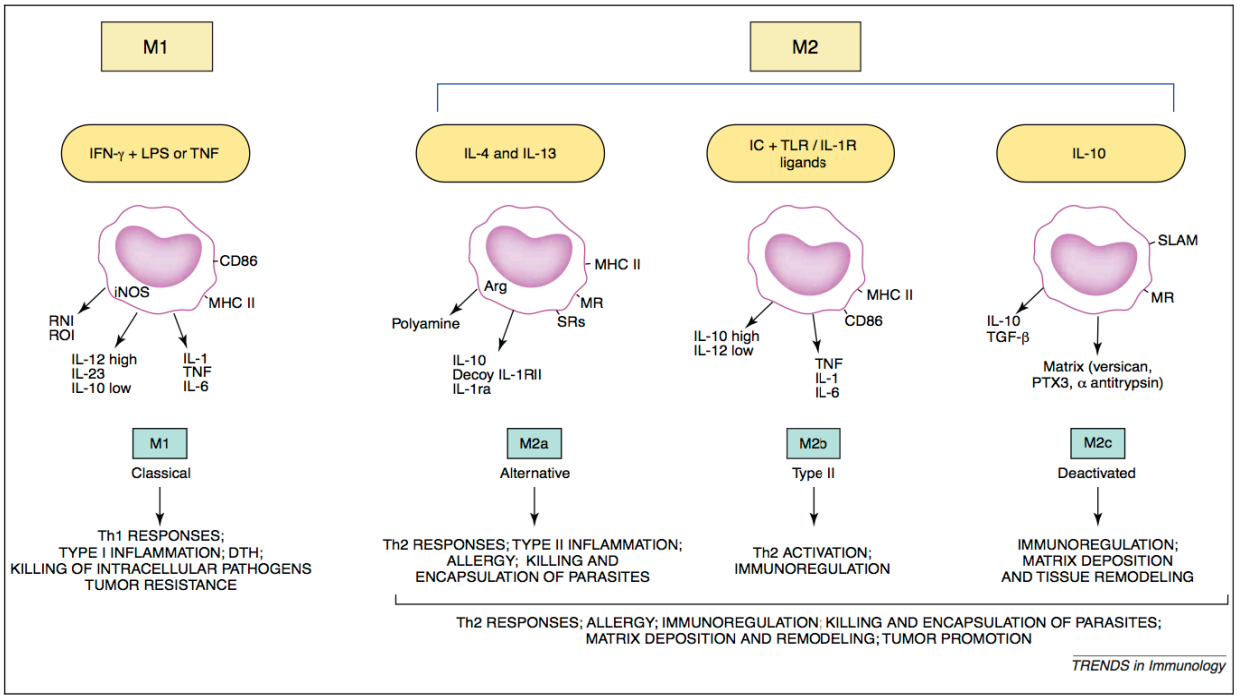


Figure 11: Induction and functional properties of polarized macrophages. The environmental-derived signal is responsible for the acquisition of different functional properties of macrophages. M1 macrophages have antimicrobial and antitumoral properties and are induced by IFNγ and LPS, while M2 macrophages are more prone to immunoregulatory and protumoral activities. IL4 and IL13 exposure drive to M2a polarization, and combined exposure to immune complexes and TLR or IL1R agonist differenciate macrophages into M2b. These types have immunoregulatory functions and drive Th2 immune response. IL-10 induces M2c phenotype whereby macrophages adopt an anti-inflammatory and wound healing profile. DTH, delayed-type hypersensitivity; IC, immune complexes; MR, mannose receptor; PTX3, the long pentraxin PTX3; RNI, reactive nitrogen intermediates; ROI, reactive oxygen intermediates; SLAM, signaling lymphocytic activation molecule; SRs, scavenger receptors; TLR, Toll-like receptor. (source: Mantovani et al. 2004)

in presence of IFNγ and pathogen pattern such as lipopolysaccharide (LPS). In this case, macrophages have anti-microbicidal and pro-inflammatory properties. M1 are the first line of defense against intracellular microbes and ensure Th1 polarization of $CD4^+$ T cells. On the contrary, if macrophages are activated by Th2 cytokines IL-4 and IL-13, they become alternative activated M2 macrophages. These cytokines trigger the phosphorylation of the transcription factor STAT6 that translocates to the nucleus where it induces the expression of genes, including M2 markers like *Arg1* (Arginase 1) and regulators such as peroxisome proliferator activated receptors (PPARγ and δ) that are useful to maintain the M2 phenotype (Chawla, 2010). These macrophages participate to the immune response against extracellular parasites like helminths. They can also help with the resolution of inflammation and tissue repair (Kiss *et al.*, 2013; Noora Ottman, 2012; Shapiro *et al.*, 2011)..

Subsequently, other research has shown that cytokines and factors non-associated with Th1 or Th2, such as IL-10, TGF-β or immune complexes, are able to elicit macrophages with a similar function to IL-4-induced M2 macrophages (Anderson *et al.*, 2002). Moreover, despite that some proteins such as Arg1, Fizz1 (for "Found in Inflammation Zone") (Pesce *et al.*, 2009) or Ym1 (for "Myeloid precursor in the Yolk sac") (Webb *et al.*, 2001) are generally considered as markers of M2 macrophages (Ghassabeh *et al.*, 2006; Gordon & Martinez, 2010; Ichinohe *et al.*, 2011; Raes *et al.*, 2002), no real consensus on M2 hallmarks has never been established. It has therefore been admitted that phenotypes and functions of M2 are multiple. In order to be more complete in the classification, three subdivisions of M2 macrophages have been proposed: M2a, M2b and M2c (**figure 11**). M2a are the alternative macrophages induced by Th2 cytokines IL-4 and IL-13. They participate in the killing and encapsulation of parasites, and are also implicated in allergy phenomena. M2b are the ones activated by immune complexes and TLR ligands. They exert immunomodulatory functions and drive a Th2 response. M2c are immunosuppressor macrophages activated by IL-10 (Gueirard *et al.*, 2003; Mantovani *et al.*, 2004; Vega & Corbi, 2006). This classification helps the understanding of immune mechanisms but remains a simplification of the real situation. A continuum of states probably exists, all dependent on multiple conditions.

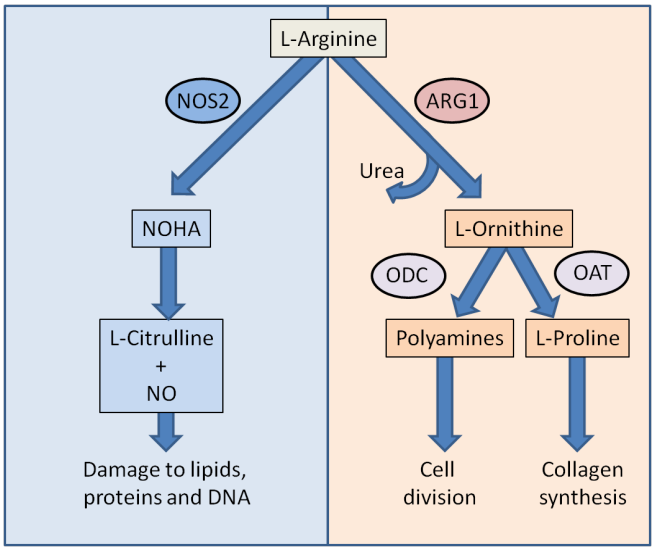


Figure 12 : Schematic view of L-arginine metabolism. Arginine is a precursor involved in two pathways. ARG1 hydrolyses arginine to L-ornithine and Urea. L-Ornithine is used for the synthesis of L-Proline by ornithine aminotransferase (OAT) and for the production of polyamines by ornithine decarboxylase (ODC). L-proline is involved in collagen production and polyamines affect cellular proliferation. Nitric oxide synthase 2 (NOS2) is responsible for the production of NOHA which is involved in the synthesis of citrulline and nitric oxide (NO). The latter generates reactive oxygen species, responsible for damage and death of pathogens. ARG1, Arginase 1; NOHA, *N*G-hydroxy-L-arginine; OAT, ornithine aminotransferase; ODC, ornithine decarboxylase. (adapted from Bronte *et al*, 2005)

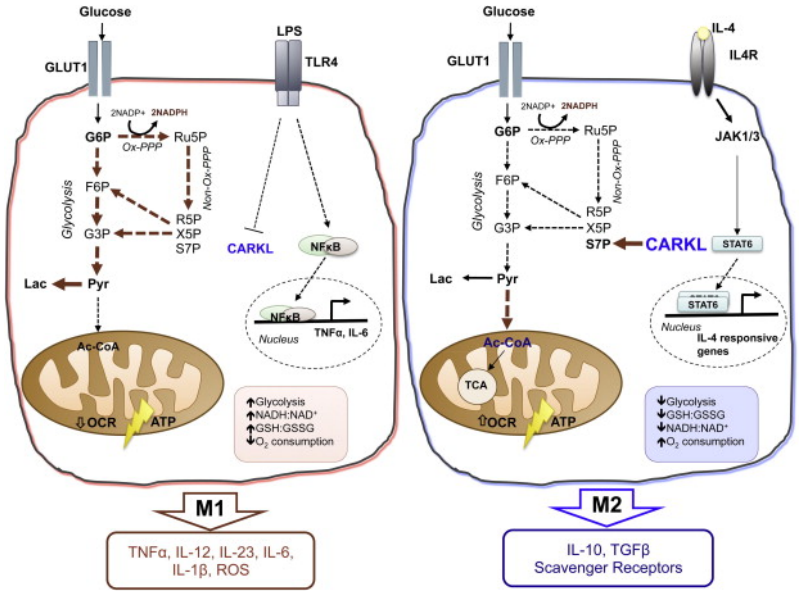


Figure 13: Reprogramming of glucose metabolism in polarized macrophages. LPS promotes the polarization of macrophages toward a pro-inflammatory M1 lineage, whereas they differentiate toward a suppressive M2 lineage in response to IL-4 and IL-13 stimulation. M1 macrophages produce pro-inflammatory cytokines such as TNFα, IL-12, IL-6 that promote inflammatory T cell differentiation, and M2 macrophages produce a higher level of scavenger receptors and immunomodulatory cytokines such as IL-10 and TGFβ. LPS signaling mediates NF-κB-mediated cytokine production and reduce the production of Carbohydrate Kinase-Like (CARKL) protein. Without CARKL, M1 metabolism is oriented through glycolysis and the oxidative pentose phosphate pathway (Ox-PPP). It leads to an increased redox potential (NADH : NAD, GSH : GSSG) and a reduced oxygen consumption rate (OCR). In M2 macrophages, CARKL promotes sedoheptulose-7-phosphate (S7P), reducing metabolism through the PPP but maintaining glycoysis and OCR.
G6P, glucose-6-phosphate; F6P, fructose-6-phosphate; G3P, glyceraldehyde-3-phosphate; Ru5P, ribuose-5-phosphate; R5P, ribose-5-phosphate; X5P, xyulose-5-phosphate. (source: Blagih *et al,* 2012)

5.2.1. M2 macrophages, a strategic plan for pathogens

Despite the diversity and plasticity of macrophages, we can use macrophages classification into M1 and M2 if we think about the general state of the cells. M2 macrophages have globally an opposite function of M1 macrophages : M1 are pro-inflammatory cells, with an important anti-microbial function, and M2 have little or no microbicidal activity, are anti-inflammatory cells, participate in the regeneration and remodeling of the tissues. To ensure their functions, M2 macrophages undergo metabolic switches that can be profitable for some intracellular pathogens such as *Francisella tularensis* or *Mycobacterium tuberculosis*; establish their niche far from the pro-inflammatory microbicidal conditions of M1 macrophages (Gordon & Martinez, 2010):

Firstly, the expression of Arg1 is promoted in M2 macrophages in mice. Arginase 1 is an enzyme of the arginine metabolism. Globally, arginine is an amino acid that can be used for the production of nitric oxyde (NO) or for the synthesis of polyamines and proline (**Figure 12**). The enzyme arginase 1 orients the arginine metabolism into the production of these two last components, which are necessary for the reparative function of M2 macrophages (cellular proliferation and collagen production, respectively) (Vega & Corbi, 2006). Without this enzyme, arginine is converted in NO, a strong microbicidal effector of the M1 macrophages. The inhibition of NO production in M2 offers a better environment for intracellular microbes (Chawla, 2010).

Secondly, glucose metabolism is differentially regulated in M1 and M2 macrophages (**figure 13**). M1 have to be able to activate rapidly their microbicidal functions, and generally to deal with hypoxic environment. Therefore, LPS signal triggers the shift of the metabolism toward the anaerobic glycolysis and oxydative pentose phosphate pathway (PPP) whereas, in IL-4 stimulated M2 macrophages, aerobic glycolysis is maintained and PPP is reduced. The elevated PPP flux in M1 macrophages generates high concentration of NADPH, which is then used for several M1 processes such as the anti-microbial NADPH-dependent respiratory burst and glutathione biosynthesis to buffer reactive oxygen species (ROS) (Haschemi *et al.*, 2012). The small quantity of available oxygen is mainly used as a substrate for NADPH oxydase during the respiratory burst (**Figure 14**). Therefore, glycolysis, using glucose as a substrate, is anaeorbic in M1 macrophages. This process produces 16 times less ATP than the aerobic glycolysis. It means that anaerobic glycolysis has to use more glucose to produce

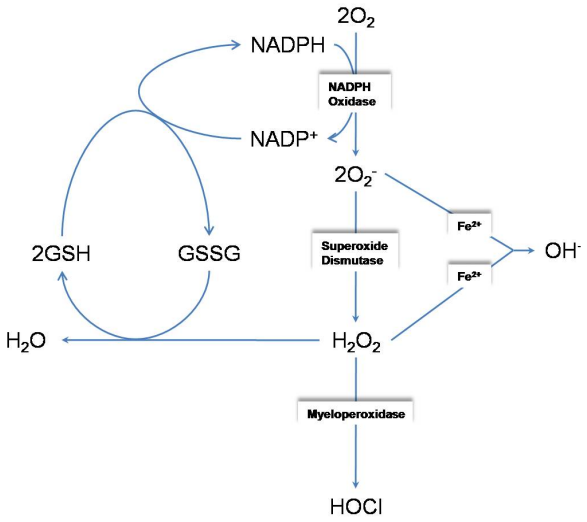


Figure 14: Respiratory burst. NADPH oxydase uses O_2 and NADPH to produce superoxyde anion (O_2^-), a Reactive Oxygen Species (ROS) and a precursor of others. Hydrogen peroxyde (H_2O_2) is produced by the enzyme superoxyde dismutase. Then H_2O_2 is converted into hypochlorus acid (HOCl) by myeloperoxydase or into hydroxyl radical (OH^-) (fenton reaction) in presence of Fe_2^+ and O_2^-. These ROS participate into the microbial killing. H_2O_2 can be reduced into H_2O by the antioxydant enzyme glutathione GSH/GSSG (reduced/oxidized). (source: www.ihtc.org)

the same amount of ATP than aerobic glycolysis. The consequence is that concentration of glucose decreases in the M1 macrophages and cannot be easily used by pathogens as a nutrient source. Moreover, surviving in hypoxia and dealing with microbicidal processes is clearly detrimental for most of pathogens. On the contrary, in M2 macrophages, the environment is aerobic, there is less anti-microbial prossesses and glycolysis supplies energy to repair the tissue while leaving nutrients available for pathogens in the cell (Biswas & Mantovani, 2012; Blagih & Jones, 2012; Nagler-Anderson, 2001; Ricardo-Gonzalez *et al.*, 2010).

Thirdly, lipid metabolism, and especially fatty acids (FAs) uptake and oxydation, is upregulated in alternative M2 macrophages. This phenomenon was first described in the context of obesity. Macrophages associated to adipose tissue are M2 polarized in healthy condition. They may have many functions in the tissue: they are anti-inflammatory notably by producing of IL-10 and inhibiting recruitment and polarization of M1 macrophages, they participate in adipose tissue expansion, and are involved in the clearance of free FAs. The last function is associated with over- expression of proteins involved in the uptake (such as the scavenger receptor and FA translocase “CD36”) and oxidation (such as Long chain acyl-CoA dehydrogenase “LCAD”) of FAs (Shapiro *et al.*, 2011). A huge excess of free FAs (FFAs) can finally disturb adipocytes (implicated in storage and release of FAs depending on the energy requirement) integrity and function, can lead to apoptosis, necrosis, M1 polarization of macrophages and inflammation that lead to insulin resistance. In a more general context, the ability of M2 macrophages to store and use fatty acid is an essential parameter to produce enough energy to fulfill their functions (Vega & Corbi, 2006). However, the storage of FAs seems also to be a source of nutrients for some pathogens. For example, *Mycobacterium tuberculosis* can derive both carbon and energy from lipids for their own metabolism (Peyron *et al.*, 2008).

Fourthly, iron metabolism changes between M1 and M2 macrophages. Iron is an essential growth factor for most bacteria. It is essential for DNA synthesis, respiration and free radical detoxification. Bacteria display mechanisms, such as the secretion of siderophores (high-affinity iron-binding organic molecule), to take it from the host environment. M1 macrophages counteract this bacterial strategy by sequestering iron. M1 repress ferroportin (Fpn) that transport iron, and induce ferritin that sequesters iron. These

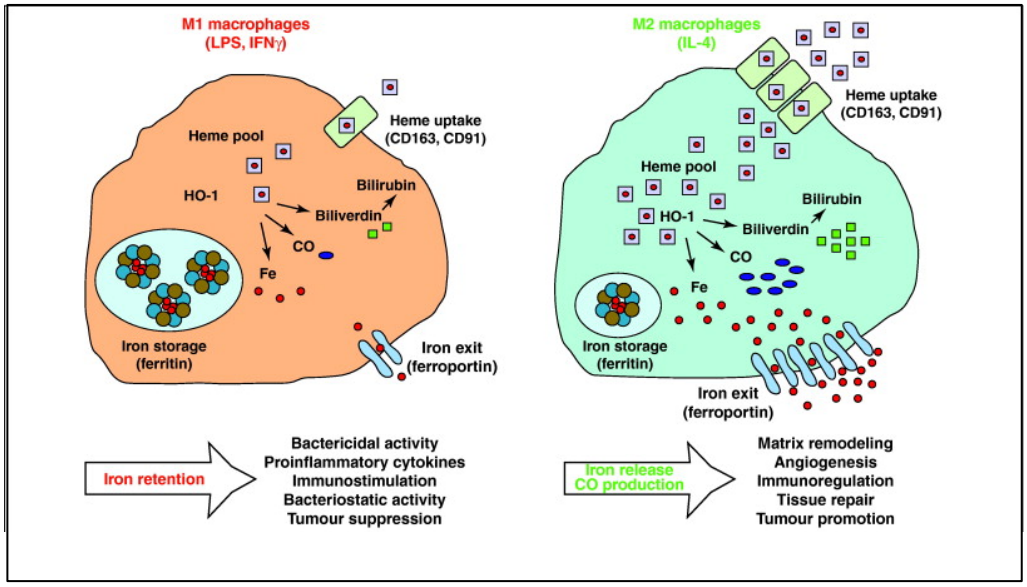


Figure 15: Iron trafficking in macrophages. The expression of ferritin, which stores intracellular iron, and ferroportin (Fpn), which exports iron from the cells, are affected by macrophage polarization. M1 macrophages display a high amount of ferritin and a reduced quantity of Fpn. This promotes storage of iron and depletion from the microenvironment. M2 macrophages are characterized by a low concentration of ferritin and high amount of Fpn. M2 have also more scavenger receptors and HO-1, which mediate heme uptake and degradation respectively. Thanks to this metabolic profil, M2 macrophages take up and degrade heme and recirculate free iron to the microenvironment. (source: Cairo *et al*. 2011)

mechanisms starve extracellular microorganisms of iron, but if these macrophages are infected, it could improve the growth of intracellular bacteria. Therefore, M1 macrophages can also limit iron available to intracellular bacteria thanks to a reduction of iron uptake by an increased expression of iron exporter Fpn (Nairz *et al.*, 2008). Besides, these macrophages produce lipocalin-2 that prevent siderophore-mediated iron capture by bacteria (Nairz *et al.*, 2007) and recruit natural resistance-associated macrophage protein 1 (NRAMP1) to the phagosomal membrane in order to export iron from the bacteria-containing phagosome (Cellier *et al.*, 2007). M2 macrophages upregulate Fpn and repress ferritin to favour the release of iron. This condition is favorable for extracellular organisms. They also promote intracellular growth of bacteria. Indeed, scavenger receptors, such as CD163 and CD91, seem to mediate heme scavenging. Internalization of heme prevent toxicity of extracellular hemoglobin derived from hemolysis during inflammation. It also reduces the pro-inflammatory and oxydant properties of heme released from hemoglobin and degraded hemoproteins (like catalase, peroxydase or cytochromes) derived from neutrophiles. The increased concentration of heme participates to the activation of the heme oxydase OH-1. This molecule contributes to the degradation of heme in three products (Gozzelino *et al.*, 2010): biliverdin, which is then converted into the anti-oxydant and anti-inflammatory bilirubin, free ferrous iron, and CO, which promotes vasodilatation and angiogenesis, and suppresses the TLR4 signaling and LPS-mediated induction of pro-inflammatory cytokines. All of this reduces inflammation and promotes tissue repair, but the iron become also available for extra- and intracellular microorganisms, helping them to survive into this host environment. (Cairo *et al.*, 2011; Mayer *et al.*, 2007; Recalcati *et al.*, 2010) (**Figure 15**).

5.2.2. Examples of persistent pathogens targeting preferentially cells with an M2-like phenotype

All the bacteria are not able to take advantage of the plasticity of macrophages. Bacteria must induce or maintain the alternative activation of macrophages to avoid their destruction by bactericidal mechanisms of M1 macrophages.

5.2.2.1. Salmonella

Box 6 gives an overview on *Salmonella*. The phenotype of the reservoir cells for the bacterium has been investigated in mouse model of *Salmonella enterica* Typhimurium

Box 6: *Salmonella,* an overview

Salmonella enterica species is a gram-negative facultative intracellular enteric bacterium responsible for a worldwide zoonosis. The transmission of the pathogen is ensured by oral ingestion of contaminated water, particularly frequent in countries where drinking water quality and sewage treatment accommodation is weak. Symptoms in humans vary from gastroenteritis to systemic infection with typhoid fever, depending on the susceptibility of the host and the infectious serovar[1]. A few percentage of patients with typhoid or patients without any symptoms become chronically infected and transmit the bacterium by their faeces and urine. These transmissions constitute real public health concerns. Moreover, treatment of the disease becomes difficult because of the increasing resistance of the bacteria (Coburn *et al.*, 2006; Guillot *et al.*, 2004; Hussell & Bell, 2014; MacLean *et al.*, 1996).

Salmonella gets into the body through the digestive tract. It resists to the acid environment of the stomach and infection is established later in the small intestine. After a passage through the M cells, *Salmonella* serovars that cause gastroenteritis and diarrhea, such as *Salmonella enterica* Typhimurium in humans or cattle, infect preferentially phagocytes in the lamina propria. The infection is self-limiting and usually does not pass beyond the mesenteric lymph node. Host adapted serovars such as *Salmonella enterica* Typhi in humans or cattle are responsible for systemic typhoid fever and can cause persistent infection within the host. After a passage through the M cells, this serovar is able to target dendritic cells and macrophages to reach mesenteric lymph nodes (MLNs) through lymphatic and blood vessels. Then, they are able to disseminate systemically through spleen, liver, gall bladder or bone marrow. The pathogen manipulates the host response by secreting effector proteins into the host cells and persists in *Salmonella* containing vacuole (SCV) in phagocytes of the MLNs, bone marrows and gall bladder for life. Sometimes, *Salmonella* can reappear on the mucosal surfaces of the intestinal tract and shedding can release it for new contaminations (Ruby *et al.*, 2012; Stumbles *et al.*, 1998; Young *et al.*, 2002).

[1] Serovar is a subdivision of a species or subspecies on the basis of antigenicity (http://medical-dictionary.thefreedictionary.com)

persistence. The most common site of chronic retention is the MLNs and at least 80 % of bacteria in these lymph nodes persist in Monocyte/Macrophage 2 (MOMA-2) expressing macrophage (Monack *et al.*, 2004). Macrophages seem also to be the predominantly infected cells in the liver (Richter-Dahlfors *et al.*, 1997), and the spleen (Salcedo *et al.*, 2001). CD18- expressing phagocytes (Macrophage or DC) are targets of *Salmonella* in the blood (Fang *et al.*, 1999). More recently, the phenotype of the infected macrophages has been investigated and the bacterium is preferentially associated with M2 macrophages (positive for M2 markers CD301, Ym1 and Arg1) at later stages of a murine infection (Eisele *et al.*, 2013). Researchers have demonstrated that the M2 marker PPARδ is required for bacterial peristence and that the pathogen actively induces its upregulation. This regulator is necessary to increase intracellular glucose, a crucial carbon source essential for the replication of *Salmonella*. Moreover, anti-inflammatory cytokines are also required in the environment of the bacteria to ensure its establishment and the maintenance of the chronic infection (Eisele *et al.*, 2013). It is not excluded that other cell types than M2 macrophages could play a role in the infection of *Salmonella* but M2 macrophages are the dominant target during persistent infection.

5.2.2.1. Mycobacterium tuberculosis

General traits of *M. tuberculosis* are presented in **Box 7**. Once inhaled and trapped by the alveolar macrophages of the lungs, the bacillus enters in a proliferative phase with an exponential growth. It corresponds to the initiation of the granuloma formation: infected macrophages, dendritic cells and non-infected macrophages aggregate to form a nascent granuloma (Holt *et al.*, 1993; Russell *et al.*, 2009; Wolf *et al.*, 2007). These cells also contribute to the development of a protective Th1 immunity. $CD4^+$ T cells participate to the enlargement of the granuloma by recruitment of other cell types such as lymphocytes and fibroblasts, and activate the secretion of reactive nitrogen and oxygen species by macrophages. At this period, when immunity begins to control the infection, bacterial load is highly reduced and the remaining bacteria enter in a non replicative persistence (Co *et al.*, 2004). In mature granuloma, secretion of TNFα and TGF-β by T cells and macrophages takes part in the integrity of the granuloma by mediating the formation of a fibrous capsule. T lymphocytes are expelled in the periphery and blood vessels decrease in the center of the structure. Oxygen content becomes low in the center and activated macrophages

Box 7: *Mycobacterium Tuberculosis*

Mycobacterium tuberculosis is an aerobe intracellular bacillus and the causative agent of tuberculosis. The disease is responsible for 9 millions new cases and 2 million deaths every year. We can estimate that one third of the population is contaminated but only 2 – 3 % develop symptoms during their life span (World Health Organization, 2009). The aerosol route ensures the transmission of the pathogen. Droplets containing bacteria expelled by infected host are engulfed by alveolar macrophages of the receiver host's lungs (Kaufmann, 2001). It uses multiple strategies to survive and replicate in its host. It avoids the maturation of the phagosome and its acidification and it resists to the antimicrobial agents. It is also able to manipulate the immune response to its advantage; the balance between the stimulation of the immune response and its inhibition is essential for mycobacterium to establish its replicative niche on the long term (Ramakrishnan, 2012). On the one hand, it limits the onset and the importance of the pro-inflammatory responses: it has been shown for example that it interferes with the responses to IFNγ or that it reduces its detection by TLR2 and the subsequent activation of macrophages (Madan-Lala *et al.*, 2011). On the other hand, *M. tuberculosis* stimulates the inflammation by inducing the recruitment of macrophages to initiate the formation of granulomas that are supposed to prevent dissemination of bacteria and limit their number in lungs but that can be beneficial for bacteria in some conditions (Davis & Ramakrishnan, 2009).

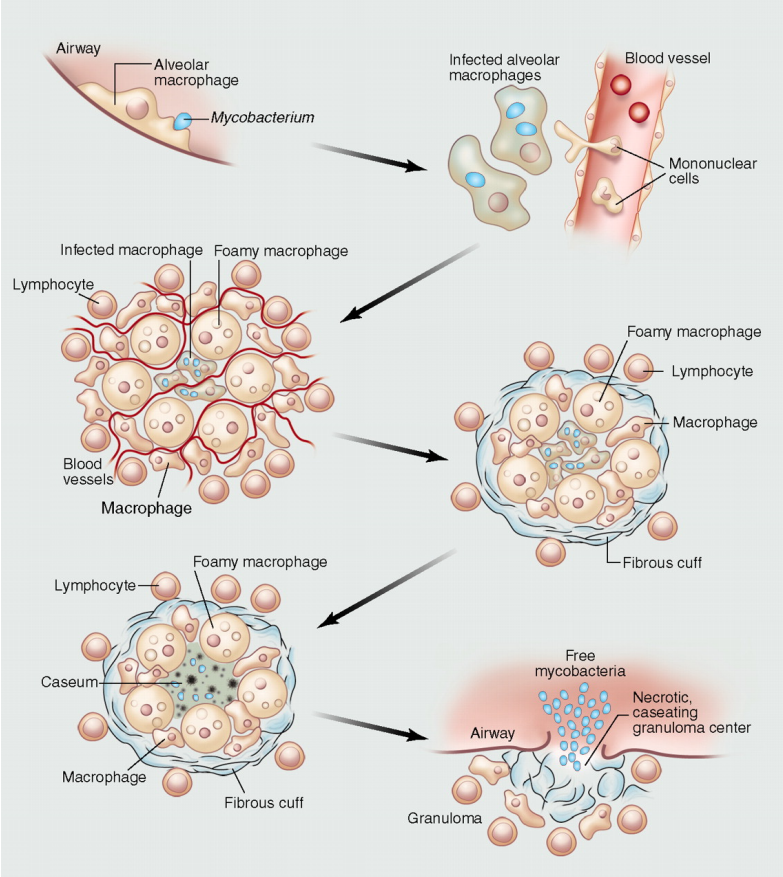


Figure 16: Foamy macrophages and the progression of the human tuberculosis granuloma. (source: Russel, D *et al*. 2009)

progressively die by apoptosis and necrosis. It leads to accumulation of debris named caseum. The apoptosis of the cells is associated with killing of contained bacteria (Dheda *et al.*, 2005). In most infected people, granulomas keep their structural integrity, do not evolve and can even resolve. However, in some cases, they become a place that favors bacterial persistence and dissemination; it happens when *Mycobacterium* manipulates the host cells to promote differentiation of alveolar macrophages into foamy macrophages that are lipid rich and display an M2-like phenotype (see **box 8** to have more information about these cells).

5.1. Brucella persistence… what is going on?

Brucella is a non-spore forming bacterium. Up to now, persister cells have not been discovered but their presence is not excluded because of the chronicity in the host. As explained in the chapter 2, *Brucella* displays a large panel of mechanisms to survive in the host: it is adapted to the harmful environment, mainly by avoiding the immune effectors, and it modifies the environment by manipulating the intravacuolar trafficking of the host cell. These strategies offer to *Brucella* the opportunity to establish a preferential niche where it persists and replicates. Chronic *Brucellosis* is far from being understood but persistence is a fact described in the literature. For example, calves infected by *Brucella abortus* in utero or by ingestion of contaminated milk can acquire a persistent infection that will stay latent until a reactivation during the pregnancy, generating abortion (Wilesmith, 1978; terHuurne *et al*., 1993). In humans, approximately 10 % of patients experience a relapse after antibiotic treatment. The phenomenon frequently happens within the first 6 months after the treatment but some cases of reactivation of the disease have been reported years later (OnderOgredici *et al.*, 2010). Symptoms of the chronic disease can be multiple, from muscular complications to neurological issues, suggesting that *Brucella* is able to survive in various environment. Studies of the infection in the mouse model confirm this versatility: spleen is known to be chronically infected (Vitry *et al.*, 2012), and bioluminescent imaging has permitted to see *Brucella* in joints, tail, extremities and submandibular regions weeks after i.p. infection (Rajashekara et al., 2005). But very little is known regarding preferential infected cells in chronically infected regions.

Box 8 : Foamy macrophages

The formation of foamy macrophages during *Mycobacterium tuberculosis* infection starts by progressive accumulation of lipid bodies by alveolar macrophages (Cáceres *et al.*, 2009). Actually, at the beginning of the infection, activation of TLRs by the PAMPs and the presence of pro-inflammatory signals, such as TNF and IFNγ, are responsible for classical M1 polarization of macrophages. It seems that some of these signals also trigger the internalization of lipids (cholesterol, triacylglycerides and phospholipids) into the pro-inflammatory macrophages that form lipid droplets (also referred to as lipid bodies) in the endoplasmic reticulum leaflet (Russell *et al.*, 2009). These lipid bodies have a role in regulating host response to infection by modulating inflammatory mediators (D'Avila *et al.*, 2008; Saka *et al.*, 2012). It also appears that the pathogen is able to take advantage of these lipid droplets because they can constitute a nutrient source for bacteria notably to synthesize their cell wall (Russell *et al*, 2009). For example, an *in vitro* study has shown that *Mycobacterium* accumulates lipids as intracytoplasmic lipid inclusions (ILI) thanks to a fusion between lipid bodies of the host cell and *Mycobacterium*-containing phagosome. At this time, the bacterium undergoes an elongation but does not divide. Lipid accumulation is intimacy correlated with the phase of latency and persistence of bacteria (Caire-Brändli *et al*, 2014). However, long persistence of *Mycobacterium* in these lipid-rich cells does not seem to be achieved in a pro-inflammatory environment. Indeed, it has been shown that the lipid-rich macrophages progressively lose their ability to phagocytose and to activate bactericidal functions (Peyron *et al.*, 2008). Reduction of pro-inflammatory activity of these macrophages combined with a lipid-rich environment could form a secure reservoir for the pathogen.

The reduction of pro-inflammatory activity of macrophages is often associated with the M2 phenotype. Some clues lead us to think that foamy macrophages are indeed M2 cells. For example, a study in mice has shown that production of iNOS by alveolar macrophages decreases during the chronic phase of the infection while expression of Arg1 increases. Moreover, there is production of IL-4 in broncho-alveolar fluid of chronically infected mice by *Mycobacterium tuberculosis* (Redente *et al*., 2010). However, foamy macrophages also express markers characteristic of other cell type, such as DEC205 and CD11c that are typical of dendritic cells (Ordway et al., 2005). Foamy macrophages are anti-inflammatory, lipid-rich and Arg1-expressing cells like alternative activated macrophages (M2) but seem to have their own characteristics that make them a cell type aside.

Besides their role in persistence of the pathogen, foamy macrophages could be used by the pathogen for later dissemination; it seems that granulomas containing these cells can have a compromised fibrous capsule and can eventually release caseus material discharging infectious bacteria into surrounding blood vessels and airways to allow the reinfection of macrophages and a rapid expansion of the colonization (Co *et al.*, 2004; Dheda *et al.,* 2005; Russel *et al.,* 2009; Stumbles *et al.,* 1998). **Figure 15** shows a schematic evolution of these structures during a *Mycobacterium tuberculosis* infection (Russel *et al.*, 2009).

Our team has investigated infected cells in the spleen at 12 days in an i.p. model and has shown that bacteria are located in the white pulp with two main infected populations: in close proximity of the marginal zone, we have found infected cells expressing MOMA1, a specific marker of metallophilic marginal zone macrophages, and deeply in the white pulp, infected cells are $CD11c^+$ and $Dec205^+$, both specific markers for DCs (Copin *et al.*, 2012). Other studies have shown that DCs can be a target during an infection by *Brucella* (Billard *et al.*, 2005). *In vitro* and *in vivo* studies have shown that *Brucella* is able to survive and replicate in other cell types such as endothelial cells (Baldi & Giambartolomei, 2013), fibroblasts (Detilleux *et al.*, 1990), hepatocytes (Delpino *et al.*, 2010), Hela cells (Pizarro-Cerdá *et al.*, 1998), and our team has recently demonstrated that erythrocytes can also be infected by the bacterium ((Vitry *et al.*, 2014), see appendix 3). Thus, it is not excluded that *Brucella* could infect other cell types during the chronic phase of the infection.

Recently, the research team of Tsolis has investigated for the first time the relative importance of alternative activated macrophages as a potential chronical niche for *Brucella.* They have shown that M2 macrophages are more abundant during a chronic infection than during an acute one. *In vitro*, bone marrow-derived macrophages (BMDM), treated with IL-4 to become alternative activated macrophages (AAM), support a higher level of bacterial replication than non-treated BMDM. BMDM treated with IFNγ to become M1-like macrophages, eliminate the bacterium. Finally, they have shown that the M2 marker PPARγ has to be expressed in macrophages to allow *Brucella* to acquire glucose and promote intracellular replication in AAM *in vitro* and persistence *in vivo* (Xavier *et al.*, 2013). These investigations are good clues to postulate that M2 macrophages could be the reservoir cells during a chronic *in vivo* infection, but it has not been demonstrated yet.

OBJECTIVES

Brucella melitensis is a bacterium having a particularly efficient mode of infection; by hiding and manipulating its host, it is able to partially evade the immune surveillance to promote its survival and its replication. Thanks to a balance between its strategies and the host immune defenses, *Brucella* is able to survive on the long term in its host. These past decades, research has improved our understanding of the infectious mechanisms of the pathogen and the associated protective immune response but there remains much to elucidate. Most experiments have used an intraperitoneal model of infection in mice. However, the natural routes of infection are mainly the ingestion of contaminated products and the inhalation of *Brucella* contaminated aerosol. By taking the mucosal route, *Brucella* is potentially able to infect other cellular populations and to activate other immune mechanisms. Therefore, we decided to develop an intranasal model of infection to characterize the immune response of mice against *Brucella melitensis*.

The work has been divided into two main parts:

- in the first part of the thesis, we studied the nature and the contribution of cellular populations into the control of *Brucella melitensis* infection. To achieve this objective, we compared wt and genetically deficient C57BL/6 mice for key elements of the immune system; immune actors known to control intraperitoneal *Brucella* infection, but also elements of the mucosal environment have been investigated.

- in the second part of the work, we analysed by immunohistofluorescence the phenotype of infected cells in spleen of susceptible BALB/c IL12p40-deficient mice during the persistent phase of *Brucella melitensis* infection. We evaluated a recent hypothesis that Th2-induced M2 macrophages could be the main reservoir cells for *Brucella.* For this purpose, we have investigated the impact of STAT6-deficiency on the bacterial load and the phenotype of infected cells in IL12p40-deficient mice.

If we knew what it it was we were doing,
it would not be called research, would it?
Albert Einstein

RESULTS

1. Both Th1 and Th17 cellular immunity constitute the core of protective lung immune response following intranasal Brucella melitensis infection in mice.

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In preparation for publication.

Over past decades, numerous studies using the intraperitoneal (i.p.) model of infection in mice have improved our knowledge about the host immune response against *Brucella melitensis*. It has been learned that MHCII-dependent antigen presentation to T lymphocytes elicits an essential Th1 immune response against *Brucella* and IFNγ-producing $CD4^+$ T cells are the crucial cell population that controls the infection. It has also been found that B lymphocytes, TAP1-dependent antigen presentation, and Th2 and Th17 immune responses do not play major roles in controlling growth of the brucellae in the host (Vitry *et al*., 2012). However, this widely used model is not representative of natural routes of infection. Specifically, i.p. infection bypasses the mucosal environment with its associated cell populations and effectors that could participate in the defenses against ingested or aerosolized *Brucella.*

When we began our intrasal (i.n.) study in 2010, few investigators had used the i.n. model of infection to study protective immunity against *Brucella* infections. The kinetics of dissemination of the brucellae to the lungs, spleen and liver had been reported, the presence of granulomas in the spleens and liver of infected mice had been observed (Mense *et a.l*, 2001), and the roles of TLR2, TLR4 and MyD88 (Pei *et al.*, 2012) and the function of adaptive immunity (Izadjoo *et al.*, 2000) in the host immure response had been described. But the role of specific immune cell populations and cytokines in protective immunity against *Brucella* infections had not been investigated.

Using a panel of genetically deficient mice, we have confirmed the importance of MHCII-dependent antigen presentation, Th1 immune response and IFNγ-producing $CD4^+$ T cells in the control of the infection in spleen, liver and lungs. We have also highlighted the collaboration of γδ T lymphocytes and production of IL-17A in the early control of bacteria in the lungs. The absence of B lymphocytes or TAP-1-dependent antigen presentation does not influence the efficiency of the immune defenses. We also showed that early localization in the spleen was different from what had been described after the i.p. infection. Finally, study of the humoral immune response during a second i.n. infection allowed us to conclude that in contrast with the i.p. model of infection, antibodies do not participate in the sterilizing immune response against the challenge strain. The mode of dissemination could be the reason for this main difference with i.p. model. Taken together these results highlight the importance of the route of infection for identifying the immune effectors implicated in the control of *Brucella* infections in the mouse model.

Both Th1 and Th17 cellular immunity constitute the core of protective lung immune response following intranasal *Brucella melitensis* infection in mice.

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Running title: Immune response against intranasal *Brucella* infection

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ABSTRACT

Brucella spp. are intracellular bacteria that cause brucellosis, one of the most common zoonoses in the world. *Brucella* infection has mainly been studied following intraperitoneal (i.p.) injection in mice. This protocol leads to an immediate bacterial dissemination to the spleen and bypasses all mucosal immune barriers. Since natural infection often occurs by the oral or respiratory route, mucosal vaccination may offer a way to confer local immune protection. Here, we characterize the cells and signaling pathways implicated in the development of a protective immune response following intranasal (i.n.) infection by *B. melitensis* 16M. The persistence of bacteria was analyzed in the lungs, spleen, liver and blood. Using a panel of genetically-deficient mice, we observed that $CD4^+$ T cells play a central role in the late control of *Brucella* growth in all of the analyzed tissues. Both Th1 and Th17 responses appear equally able to eliminate *Brucella* in lungs and liver, but only Th1 seems able to control the level of *Brucella* persistence in the spleen. Deficiency of $CD8^+$ T cells does not significantly impact the course of *Brucella* infection but absence of γδ T cells strongly affects the early infection control in the lungs. In contrast to the i.p. infection model where antibodies play a crucial role in protection against a secondary infection, we showed that B cells are dispensable in both primary and secondary i.n. infections. Taken together, our results demonstrated that both Th1 and Th17 cellular immunity constitute the core of the protective immune response against i.n. infection by *B. melitensis*. Identification of Th1 and Th17 cellular response as immune protective markers could help to define a rational strategy for developing an effective human vaccine against brucellosis.

INTRODUCTION

Brucella are facultative intracellular Gram-negative coccobacilli that infect humans as well as domestic and wild mammals. Human brucellosis is a zoonotic infection acquired through contact with infected animals or their products via ingestion, inhalation, or contact with conjunctiva or skin lesions (1). Although it is rarely fatal, it is a severe and debilitating chronic disease without prolonged antibiotic treatment (2). Despite significant progress, the incidence of human brucellosis remains very high in endemic areas (3) and is considered to be largely underestimated (4). In addition, *Brucella* species have been "weaponized" by several governments and are presently classed as category B threat agents (5). As the complete eradication of *Brucella* would be unpractical due to its presence in a large range of wild mammals (6, 7) and because antibiotic treatment is costly and patients frequently suffer from relapses following treatment (8), vaccination remains the more rational strategy to confer protection to populations living in endemic countries and to professionals frequently exposed to *Brucella*. Unfortunately, there is currently no vaccine against human brucellosis. All of the commercially available animal vaccines cause disease in humans (9, 10).

Live vaccines are widely accepted to be superior to inactivated vaccines for protection against brucellosis (11–13), suggesting that the intracellular persistence of *Brucella* antigens generates key signals allowing the development of protective immunity. In addition, several studies from the *Brucella* experimental model (14, 15) but also from other infectious models reviewed in (16) have demonstrated that the infection route can influence the localization, the duration and the nature of protective immune responses. However, to date, the large majority of experimental studies with live *Brucella* strains have predominantly focused on intraperitoneal (i.p.) delivery in mouse models, frequently limiting their analysis to present bacteriologic counts in organs such as the spleen and liver after challenge. Thus, in spite of the importance of aerosol exposure as a natural route of infection (17) and the potential risk of the usage of aerosolized *Brucella* spp. as a bioterror threat (5), very few studies (18–23) have

focused on the respiratory route of infection. A limited number of brucellae seem sufficient to infect humans by aerosol (24). In agreement, aerosol or intranasal (i.n.) exposure of mice (23) or macaques (25) with low (10^2–10^3) doses of virulent *B. melitensis* 16M caused pulmonary infection followed by systemic infection of the spleen and liver. Interestingly, immune effector mechanisms controlling *Brucella* growth in the lungs, liver and spleen appear to differ depending upon the route of infection used in the mouse model (18), suggesting that caution is warranted in interpretation of the significance of host immune effectors components previously identified using the i.p. route of infection and spleen colonization as the measure of *Brucella* virulence.

To increase our understanding of the nature of protective mechanisms induced by i.n. administration of live *Brucella* vaccines, we analyzed the protection levels and the immune responses elicited in several host compartments (e.g. the lungs, spleen, liver and blood) and at different times after primary and secondary i.n. infection with virulent *B. melitensis* 16M. In this model, using a large panel of genetically deficient C57BL/6 mice, we compared the importance Th1, Th17 and $CD4^+$T, $CD8^+$T, $\gamma\delta^+$T and B cells in the early and late control of *Brucella* growth. On the whole, these results identify both Th1- and Th17-based cellular immunity as potential markers of protective immune response against i.n. infection by *B. melitensis*, and could thus help to develop a rational strategy to identify protective live vaccines against human brucellosis.

MATERIALS AND METHODS

Ethics Statement

Procedures of this study and mice handling is conform with current European legislation (directive 86/609/EEC) and the corresponding Belgian law "Arrêté royal relatif à la protection des animaux d'expérience du 6 avril 2010 publié le 14 mai 2010". The Animal Welfare Committee of the Université de Namur (UNamur, Belgium) has reviewed and approved the complete protocol (Permit Number: 05-558).

Mice and reagents

Wild-type C57BL/6 mice were acquired from Harlan (Bicester, UK). IFNγR$^{-/-}$ C57BL/6 mice (26), IL-12p35$^{-/-}$ C57BL/6 mice (27), IL12p40$^{-/-}$ C57BL/6 mice (28)from Dr. B. Ryffel (University of Orleans, France). IL17RA$^{-/-}$ C57BL/6 mice (29), IL23p19$^{-/-}$ C57BL/6 mice (30) from Dr. K. Huygen (Belgian Scientific Institute for Public Health, Bruxelles, Belgium). TAP1$^{-/-}$ C57BL/6 mice (31), MHCII$^{-/-}$ C57BL/6 mice (32) from Jörg Reimann (University of Ulm, Ulm, Germany). RAG1$^{-/-}$ C57BL/6 mice (33) from Dr. S. Goriely (Université Libre de Bruxelles, Belgium). AID$^{-/-}$ C57BL/6 mice (34) from Dr. H Jacobs (The Netherlands Cancer Institute, The Netherlands).). CD3ε$^{-/-}$ C57BL/6 mice (35), IL-1R$^{-/-}$ (36), CCR2$^{-/-}$ (37) and MuMT$^{-/-}$ (38) C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). All wild-type and deficient mice used in this study were bred in the animal facility of the Gosselies campus of the Université Libre de Bruxelles (ULB, Belgium).

We used a strain of *Brucella melitensis* 16M stably expressing a rapidly maturing variant of the red fluorescent protein DsRed (39): the mCherry protein (mCherry-Br), under the control of the strong *Brucella* spp. promoter, PsojA. Construction of the mCherry-Br strain has been described previously in detail (40). It grows in biosafety level III laboratory facilities. Cultures were grown overnight with shaking at 37 °C in 2YT media (Luria-Bertani

broth with double quantity of yeast extract) and were washed twice in RPMI 1640 (Gibco Laboratories) (3500xg, 10 min.) before the inoculation in mice.

Mice infection and challenge

Mice were anaesthetized with a cocktail of Xylasin (9mg/kg) and Ketamin (36 mg/kg) in PBS before being inoculated intra-nasally (i.n.) with $2x10^4$ CFU of wild type or mCherry-expressing *B. melitensis* in 30 µL of RPMI. Control animals were inoculated with the same volume of RPMI. Plating serial dilutions of inoculums validated the infectious doses. At selected time after infection, mice were sacrificed by cervical dislocation. Immediately after sacrifice, spleen, liver and lung cells were collected for bacterial count, flow cytometry and/or microscopic analyses. The intra-peritoneal infection made as a comparison with our i.n. model was previously described (40).

Bacterial count

Spleens, livers and lungs were crushed and transferred in PBS/0.1% X-100 triton (Sigma). For bacterial counts in the blood, 75 µl of blood was collected from the tail with heparinated capillaries at selected time points and diluted in PBS/0.1% X-100 triton (Sigma). We performed successive serial dilutions of the triton lysate in RPMI to get the most accurate bacterial count and plated them onto 2YT medium. The CFU were counted after 5 days of culture at 37°C. The CFU were counted after 5 days of culture at 37°C.

Cytofluorometric analysis

As previously described (41), spleens were harvested, reduced in small pieces and incubated for 30 minutes at 37°C with a mix of DNAse I fraction IX (Sigma-Aldrich Chimie SARL, Lyon, France) (100 µg/ml) and 1.6 mg/ml of collagenase (400 Mandl U/ml). Spleen cells were washed and filtered before being incubated in saturating doses of purified 2.4G2 (anti-mouse Fc receptor, ATCC) in 200 µl PBS 0.2% BSA 0.02% NaN3 (FACS buffer)

during 20 minutes at 4 °C to prevent antibody binding to Fc receptor. Various fluorescent mAb combinations in FACS buffer were used to stain 3-5x106 cells. We acquired the following mAbs from BD Biosciences: Fluorescein (FITC)-coupled 145-2C11 (anti-CD3ε), Phycoerythrin (PE)-coupled RM4-5 (anti-CD4), Phycoerythrin (PE)-coupled 53-6.7 (anti-CD8α), Biotin-conjugated RB6-8C5 (anti-Gr1), streptavidin Phycoerythrin (PE), Fluorescein (FITC)-coupled M1/70 (anti-CD11b), Fluorescein (FITC)-coupled 1A8 (anti-Ly6G), allophycocyanin (APC)-coupled 1-A/1-E (anti-MHCII), allophycocyanin (APC)-coupled BM8 (anti-F4/80). The cells were analyzed on a FACScalibur cytofluorometer. Dead cells and debris were eliminated from the analysis according to size and scatter.

Intracellular cytokine staining

To guarantee an intracellular staining, after DNase-collagenase treatment, spleen cells were incubated for 4 h in RPMI 10% FCS with 1 μl/ml Golgi Stop (BD Pharmingen) at 37°C, 5% CO2. After washing with FACS buffer and staining for cell surface markers, cells were fixed in PBS/1% PFA for 15-20 min at 4 °C. They were then permeabilized for 30 min using a saponin-based buffer (10X Perm/Wash, BD Pharmingen in FACS buffer) and stained with allophycocyanin (APC)-coupled XMG1.2 (anti-IFN-γ; BD Biosciences) or allophycocyanin APC-coupled eBio17B7 (anti-IL17a; BD Biosciences). After final fixation in PBS/1% PFA, cells were analyzed on a FACScalibur cytofluorometer. No signal was detectable with control isotypes.

Immunofluorescence microscopy

Spleens and livers were fixed for 4 hours at room temperature in 2% paraformaldehyde (pH 7.4), washed in PBS, and incubated overnight at 4°C in a 20% PBS-sucrose solution. Lungs were submitted to the same treatments at room temperature and under vacuum. Tissues were then embedded in the Tissue-Tek OCT compound (Sakura), frozen in liquid nitrogen, and cryostat sections (5 nm) were prepared. For staining, tissue sections were

rehydrated in PBS and incubated in a PBS solution containing 1% blocking reagent (Boeringer) (PBS-BR 1%) for 20 minutes before being incubated overnight in a PBS-BR 1% containing any of the following mAbs or reagents: DAPI nucleic acid, Alexa Fluor 350 or 488 phalloidin (Molecular Probes), Alexa Fluor 647-coupled BM8 (anti-F4/80, Abcam), Alexa Fluor 647-coupled M1/70 (anti-CD11b), Alexa Fluor 647-coupled HL3 (anti-CD11c), Biotin-coupled (anti-Gr1) or IgG M-19 (anti-NOS2) to stain the cells of interest. Biotin-coupled anti-Gr1 mAb and anti-NOS2 polyclonal antibodies were detected using Alexa 350-coupled streptavidin Alexa Fluor 488-coupled goat anti-rabbit IgG (Molecular Probes) in PBS-BR 1%, respectively. Slides were mounted in Fluoro-Gel medium (Electron Microscopy Sciences, Hatfield, PA). Labelled tissue sections were visualized with an Axiovert M200 inverted microscope (Zeiss, Iena, Germany) equipped with a high-resolution monochrome camera (AxioCam HR, Zeiss). Images (1384x1036 pixels, 0.16µm/pixel) were acquired sequentially for each fluorochrome with A-Plan 10x/0.25 N.A. and LD-Plan-NeoFluar 63x/0.75 N.A. dry objectives and recorded as eight-bit grey-level *.zvi files. At least 3 slides per organ were analyzed from 3 different animals and the results are representatives of 2 independent experiments.

Lung electronic microscopy

The lung samples were fixed with glutaraldehyde 2.5 % for few hours at 4°C, rinsed in cacodylate buffer 0.1 M, pH 7.0 and cut with a LEICA vibratome VT1200S to 300 µm-thick sections. After serial dehydration in increasing concentrations of ethanol and finally acetone, samples were dried at CO2 critical point and mounted on SEM stubs. Observations were perfomed with ESEM Quanta F200 (FEI) microscope. Images were analyzed and processed by iTEM software.

ELISA

Specific murine IgM, IgA, IgG1, IgG2a and IgG3 isotypes were determined by enzyme-linked immunosorbent assay (ELISA). Polystyrene plates (Nunc 269620) were coated with heat-killed *B. melitensis* (10^7 CFU/ml). After incubation overnight at 4°C, plates were blocked for 2 hours at room temperature (RT) with 200 µl of PBS-3.65% casein. Then plates were incubated overnight at 4 °C with 50 µl of serial dilutions of the serum in PBS-3.5% casein. The sera from unimmunized mice were used as the negative control. After 4 washes with PBS, isotype-specific goat anti-mouse horseradish peroxidase conjugates were added (50 µl/well) at appropriate dilutions (anti-IgM from Sigma; anti-IgA from Invitrogen; LO-MG1-13 HRPO, LO-MG2a-9 HRPO, LO-MG3-13 HRPO from LO-IMEX). Plates were incubated for 2 hours at room temperature and washed 4 times in PBS before the addition of 100 µl of substrate solution (BD OptEiA) to each well. After 10 minutes of incubation at room temperature in the dark, the enzyme reaction was stopped by adding 25 µl/well of 2N H2SO4, and absorbance was measured at 450 nm.

Statistical analysis

We used a (Wilcoxon-) Mann-Whitney test provided by GraphPad Prism software to statistically analyze our results. Each group of deficient mice was compared to wild-type mice. We also compared each group with each other and displayed the results when required. Values of $p < 0.05$ were considered to represent a significant difference. *, **, *** denote $p<0.05$, $p<0.01$, $p<0.001$, respectively.

RESULTS

Intranasal inoculation leads to dissemination of *Brucella* in mice

In the present study, we used *B. melitensis* 16M strain to infect C57BL/6 mice by the i.n. route to investigate the nature of the protective immune responses in the lungs, spleen, liver and blood. This model has been previously used by several research groups (18, 20, 21). However, as infectious dose, origin of mice and animal facility conditions could influence the course of infection, we began by establishing the kinetics of i.n. infection under our laboratory conditions.

Following i.n. inoculation with $2x10^4$ CFU of *B. melitensis*, mice were sacrificed at 1, 3, 6, 12, 20, 28, and 50 days post infection (p.i.) (Figure 1.A). We observed that the brucellae persist at the same level in the lungs for the first 12 days p.i. before being progressively eliminated. At 28 days p.i., only rare mice displayed detectable CFU in the lungs. The dissemination of *Brucella* from the lungs to the spleen and liver took approximately 3 days. From 6 days p.i. the spleen and liver of the majority of mice displayed detectable CFU counts that reached a maximum at 12 days p.i.. Infection persisted in the spleen until the end of the experiment (50 days p.i.) but decreased from 12 days p.i. to below the limit of detection in the livers of the majority of mice. When compared to i.p. infection with the same dose of bacteria ((42) and Figure 1.B), a striking characteristic of i.n. infection was the slow dissemination from the primary site of infection to the spleens and livers and the absence of detectable brucellae in the blood (detection threshold = 10 CFU / ml).

It has been reported that *B abortus* infects mainly $CD11c^+$ $F4/80^+$ $MHCII^-$ alveolar macrophages in the lungs (43). Using a high dose ($2x10^7$ CFU) of mCherry-expressing *B. melitensis* 16M, we confirmed this observation at 1d p.i. (Supplementary Figure 1.A). After i.n. administration of $2x10^4$ CFU, flow cytometry analysis of infected lungs did not show a significant modification of the frequency of neutrophils, alveolar and interstitial macrophages until 5 days p.i. (Supplementary Figure 1.B). As previously reported by others (43), we did

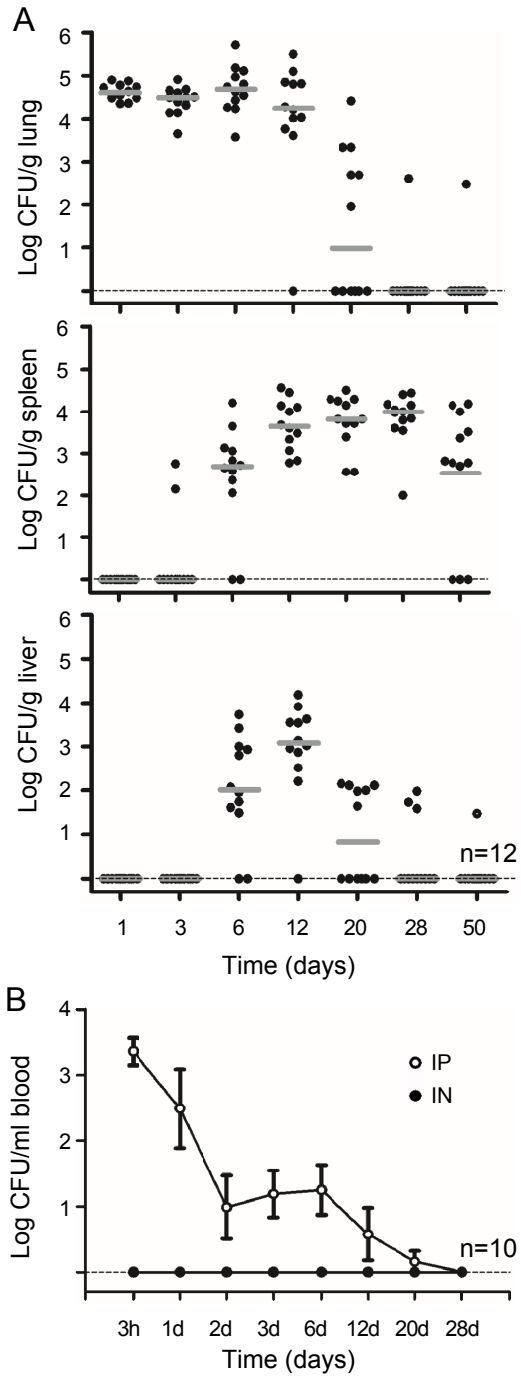

Figure 1. Course of *B. melitensis* infection in organs of C57BL/6 wild-type (wt) mice. Mice were intranasally (i.n.) injected with $2x10^4$ CFU of *B. melitensis* and sacrificed at the indicated times. **A,** The data represent the number of CFU per gram of lungs (up), spleen (middle) and liver (down). Grey bars represent the medians. **B,** The data represent the number of CFU per ml of blood. These results are representative of at minimum three independent experiments. n denotes the number of mice used for each time.

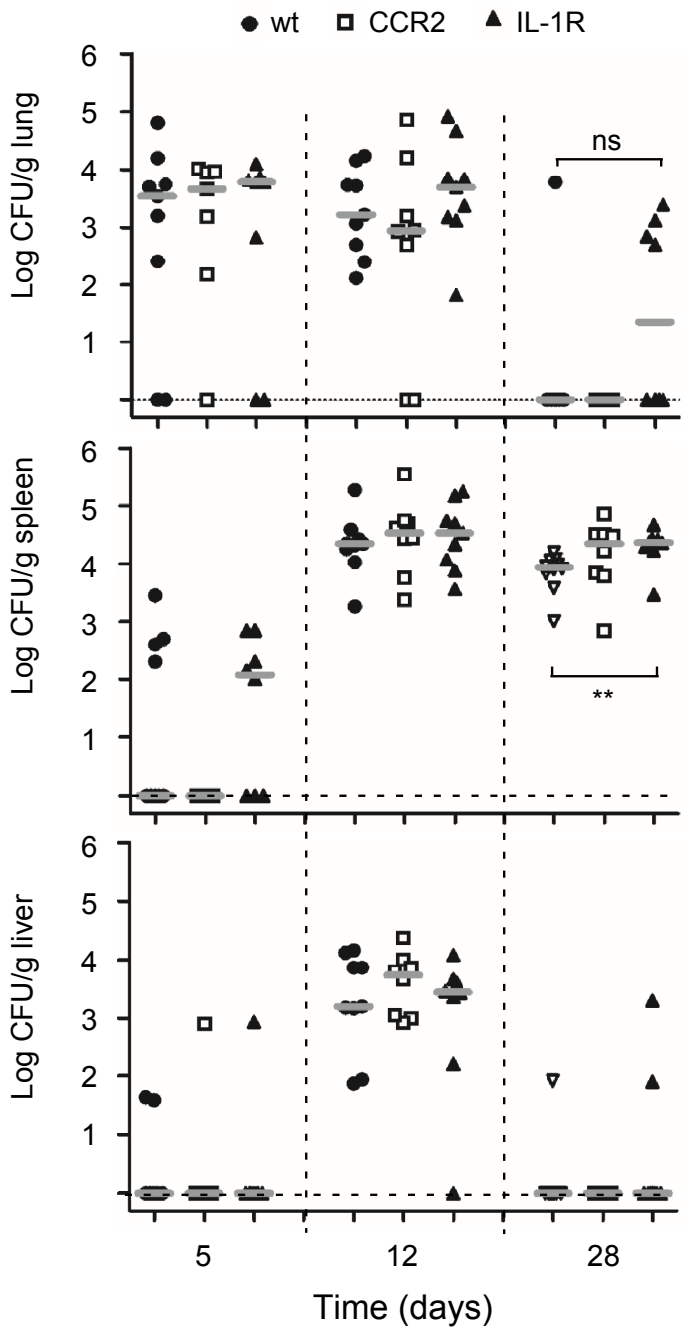

Figure 2: Course of *B. melitensis* mcherry in organs of C57BL/6 wild type, CCR2- and IL-1R-deficient mice. Mice were i.n. infected with a dose of $2x10^4$ CFU of *B. melitensis* and sacrificed at the indicated times. The data represent the number of CFU per gram of lungs (up), spleen (middle) and liver (down). Bars represent the medians. n denotes the number of mice used for each lineage at each time. These results are representative of at minimum two independant experiments. **, P<0,01; ns, non-significant.

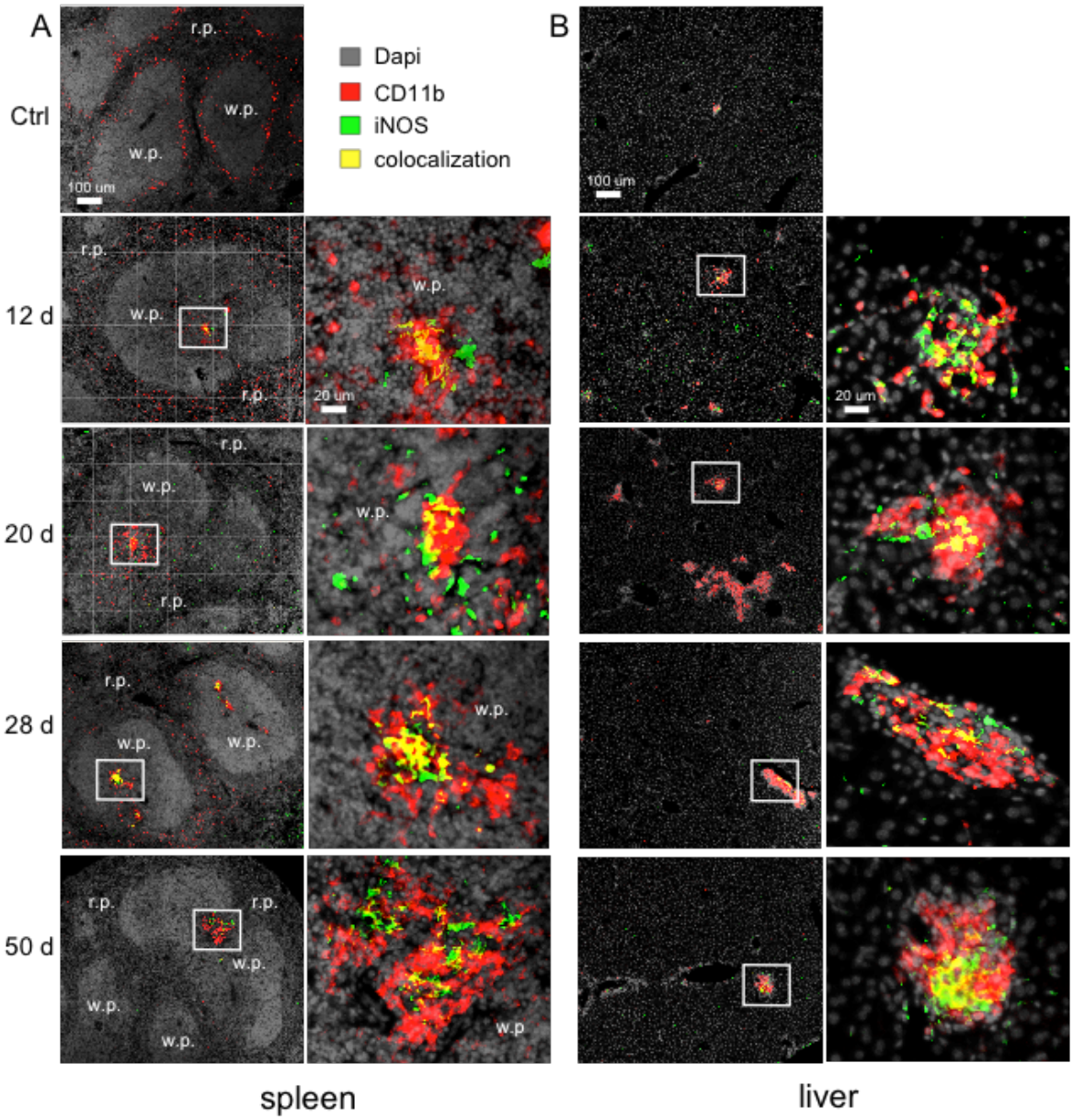

Figure 3: CD11b$^+$ granulomas in the spleen and liver of C57BL/6 wt mice. Mice were infected i.n. with a dose of 2x10^4 CFU of *B. melitensis* and sacrified at the indicated times. The panels represent the localization by immunofluorescence of CD11b$^+$ and iNOS expressing cells in spleen **(A)** and liver **(B)** from naïve (control) and infected mice since 12, 20, 28 and 50 days. Right panels of **(A)** and **(B)** are higher magnification views of the left panels **(A)** and **(B)**, respectively. Panels are color-coded with the text for Dapi or the antigen examined. Scale bar=100 µm and 20 µm, as indicated. r.p.: red pulp; w.p.: white pulp. Data are representative of at least two independent experiments.

not observe granuloma formation in the lungs at any phase of infection (Supplementary Figure 1.C). These results suggest that the control of *Brucella* infection in mucosal lung tissue does not induce or require an inflammatory reaction. In order to test this hypothesis, we compared the course of *Brucella* infection in wild type, IL-1R$^{-/-}$ (deficient for proinflammatory cytokine IL-1 signaling) and CCR2$^{-/-}$ (deficient for monocyte recruitment) C57BL/6 mice (Figure 2). As expected, deficient mice displayed similar CFU counts in the lungs to wild-type mice during the first 12 days of infection, confirming that inflammation and monocyte recruitment are not required to control *Brucella* infection in the lungs.

In drastic contrast to what was observed in the lungs, colonization of the spleen (Figure 3.A) and liver (Figure 3.B) is accompanied by granuloma formation in i.n. infected mice which was detected by CD11b and iNOS/NOS2 staining. As expected, these granulomas were stained by rabbit anti-*Brucella* LPS (supplementary Figure 2). Interestingly, the location of the granulomas in the spleens and livers after i.n. infection was clearly different from their location after i.p. infection. As previously reported (40), following i.p. infection, granulomas were located predominately in the red pulp during the first days of infection and were found in the white pulp only after 12d. After i.n infection, granulomas appeared directly and only inside the white pulp, suggesting that the red pulp location of granulomas could be an artifact of the i.p. route of infection allowing a rapid and abrupt infection of the spleen.

Both Th1 and Th17 response contribute to the control of intranasal infection

The central role of the IFN-γ-mediated Th1 immune response in controlling spleen colonization by *Brucella* strains following i.p. infection has been well established (40, 44, 45). A weak compensatory role of Th17 in absence of Th1 in the spleen has been also reported by comparing IL-12p35$^{-/-}$ and IL-12p40$^{-/-}$ C57BL/6 mice (45). Using a battery of mice genetically deficient for key factors promoting Th1 (IL-12) and Th17 (IL-23) (Figure 4), we investigated the importance of both responses in controlling the persistence of the brucellae in the lungs, spleen and liver. We confirmed with i.n. infection the results obtained in the

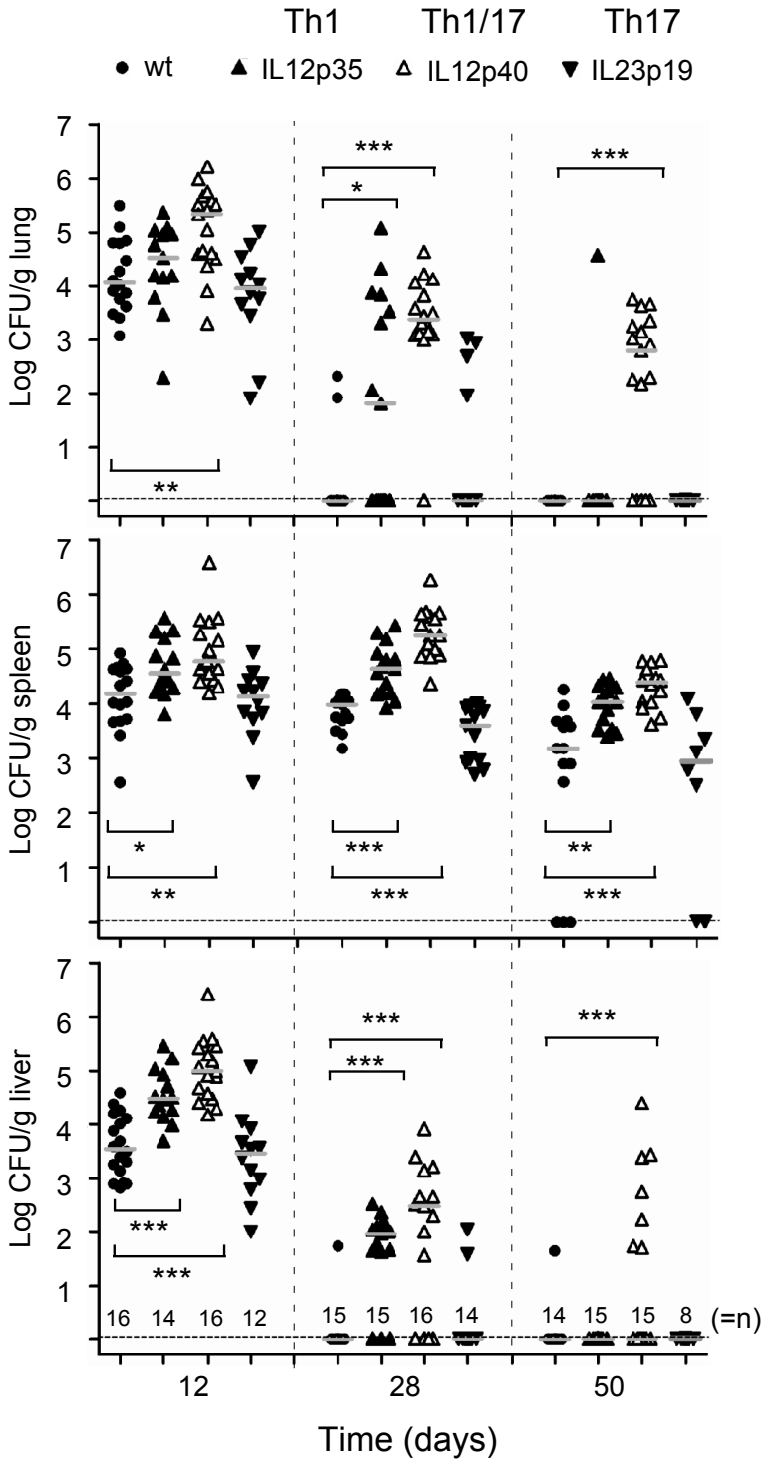

Figure 4: Course of *B. melitensis* mcherry in organs of C57BL/6 wt, p40-, p35- and p19-deficient mice. Mice were i.n. infected with a dose of $2x10^4$ CFU of *B. melitensis* and sacrificed at the indicated times. The data represent the number of CFU per gram of lungs (up), spleen (middle) and liver (down). Bars represent the medians. n denotes the number of mice used for each lineage at each time. These results are representative of at minimum two independent experiments. *, P<0,05; **, P<0,01; ***, P<0,001.

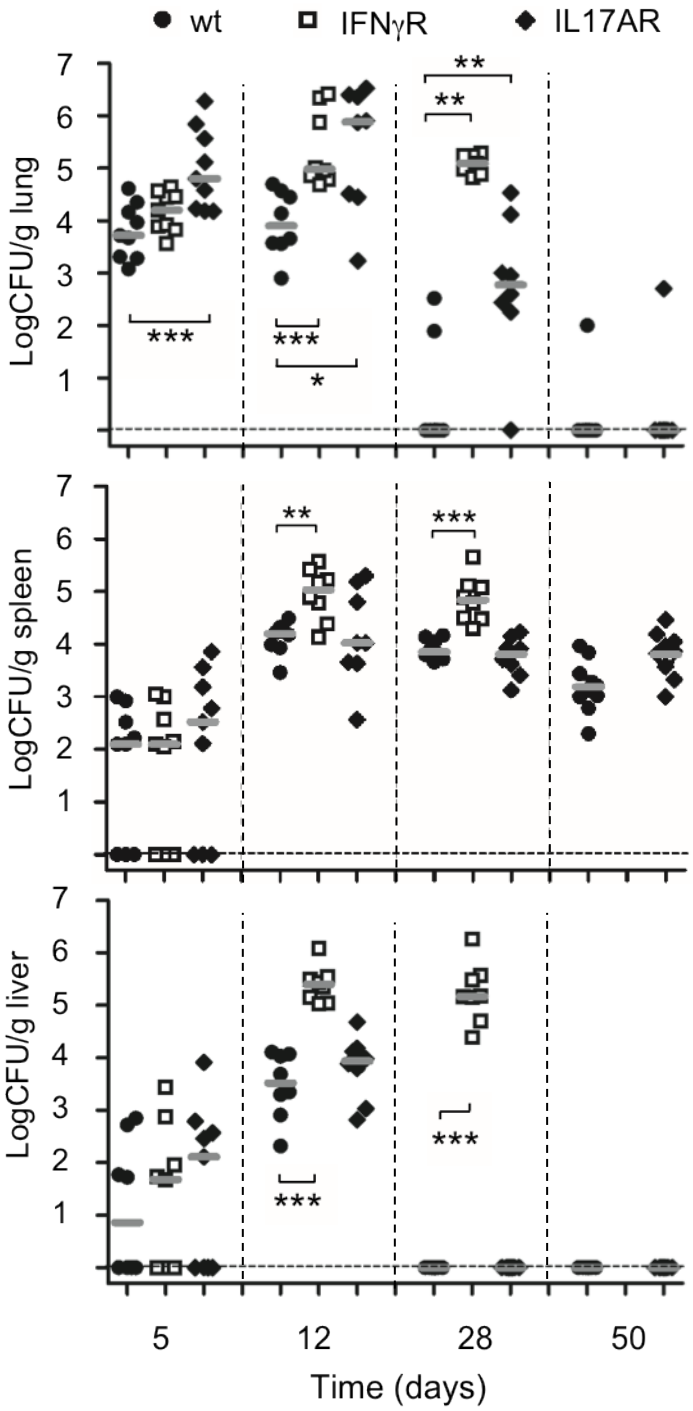

Figure 5: Course of *B. melitensis* mcherry in organs of C57BL/6 wt, IFNγR- and IL-17AR-deficient mice. Mice were i.n. infected with a dose of $2x10^4$ CFU of *B. melitensis* and sacrificed at the indicated times. The data represent the number of CFU per gram of lungs (up), spleen (middle) and liver (down). Bars represent the medians. n denotes the number of mice used for each lineage at each time. These results are representative of at minimum two independant experiments. *, P<0,05; **, P<0,01; ***, P<0,001.

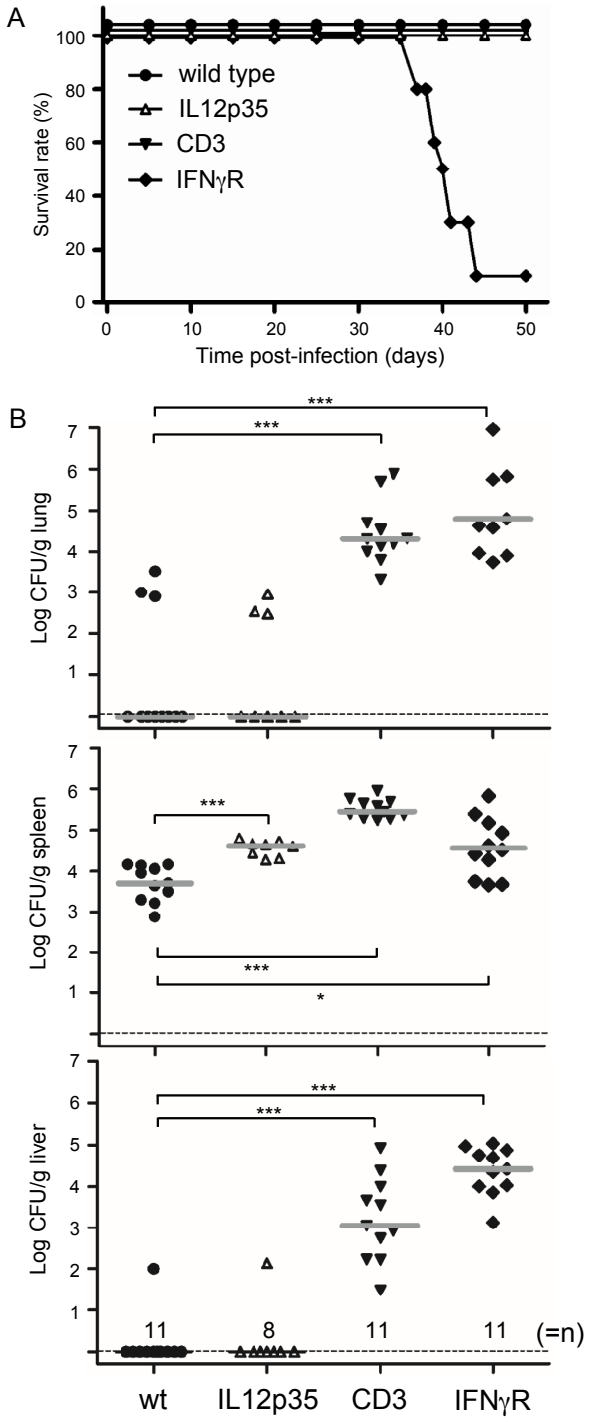

Figure 6: Comparison between the *B.melitensis* infection of C57BL/6 wt, IL12p35-, CD3-and IFNγR-deficient mice. Mice were i.n. injected with $2x10^4$ CFU of *B. melitensis;* **A,** Survival curve of the four lineages of infected mice. **B,** mice were sacrificed at 35 days post-infection. The data represent the number of CFU per gram of lung (up), spleen (middle) and liver (down). Grey bars represent the medians. n denotes the number of mice used for each lineage. These results are representative of at minimum two independant experiments. *, P<0,05; **, P<0,01; ***, P<0,001.

spleen after i.p. infection. IL-23p19$^{-/-}$ mice (Th17^{-}) displayed CFUs count in the spleen similar to wild type mice at any time tested p.i.. In contrast, IL-12p35$^{-/-}$ (Th1^{-}) present higher CFU counts during the entire course of infection. IL-12p40$^{-/-}$ mice, deficient for IL-12 and IL-23 (Th1^{-} and Th17^{-}), displayed higher CFU counts at later time p.i..

In drastic contrast to what was obseved in the spleen, IL-12p35 deficiency had only a transient impact on the capacity of the brucellae to colonize the lungs and liver. At 50d p.i., these mice eliminate *Brucella* like wild-type mice. Only IL-12p40 deficiency, affecting both Th1 and Th17 response, leads to high persistence in the lungs and liver. IL-23p19 deficiency did not affect the course of infection. These results showed that the immune requirements to control *Brucella* infection are tissue-specific, and that both Th1 and Th17 responses are equally able to control *Brucella* in the lungs and liver.

As IFN-γ and IL-17 are the signature effector cytokines of the Th1 and Th17 immune responses, respectively, we compared the impact of IFN-γR and IL-17RA deficiencies on the course of i.n. *Brucella* infection. As previously reported by others (46–48), absence of functional IFN-γ signaling led to high CFU counts in all of the tested organs (Figure 5) and mortality after 35d p.i. (Figure 6.A). In comparison, the impact of IL-17 signaling deficiency appears to be limited to higher CFU counts in the lungs early in the infection (Figure 5) without any associated mortality (data not shown). Interestingly, the impact of IL-17R deficiency is already marked at 5d p.i.. At this time, IFN-γ signaling deficiency has no impact in the lungs, suggesting that the Th17 response starts before the Th1 response in the lungs, and constitutes the first line of defense against *Brucella* in this organ.

High mortality in IFN-γ signaling deficient mice in response to *Brucella* infection is associated to dramatic neutrophilia

The fatal course of *Brucella* infection in all mice displaying a deficiency in IFN-γ signaling (IFN-γ$^{-/-}$ (46, 48), IRF1$^{-/-}$ (47) mice) supports the idea that IFN-γ plays a pivotal role

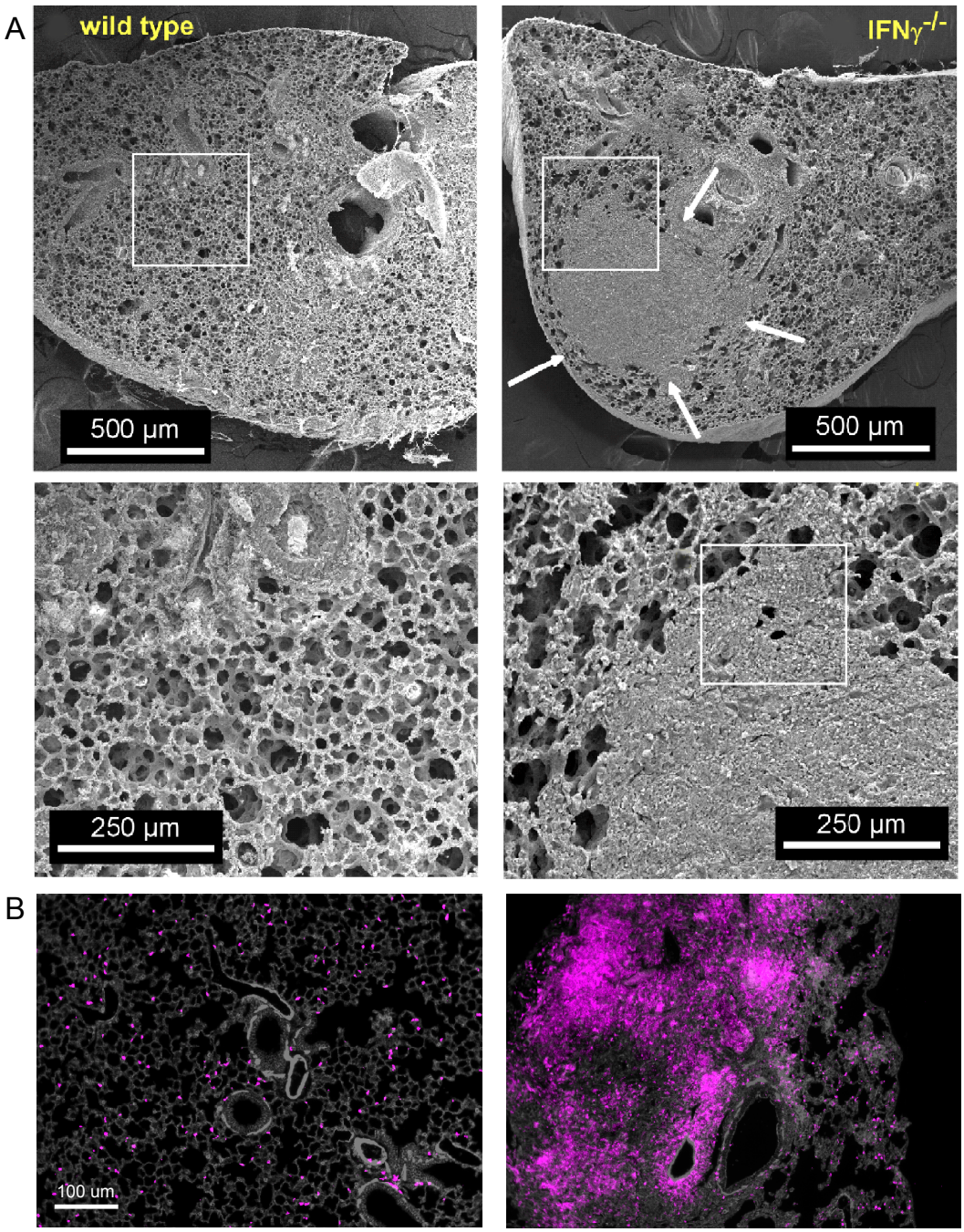

Figure 7: Electronic and fluorescent microscopic analysis of lungs of C57BL/6 wt and IFNγR-deficient mice. Mice were i.n. injected with $2x10^4$ CFU of *B. melitensis* and killed 35 days post-infection; **A,** Electronic microscopic analysis of lung in infected wt (left) and IFNγR-deficient mice (right). White arrows indicate a large zone devoid of alveoles, The two lower panels are higher magnification view. **B,** Immunohistofluorescence of lung in wt (left) and IFNγR-deficient mice (right). Panels are color-coded with the text for phalloidin or the antigen examined (Gr1). Scale bar=500, 250 and 100 µm, as indicated. Data are representative of at least two independent experiments.

in mediating a protective immune response. However, the complete absence of mortality in RAG1$^{-/-}$ (45), MyD88$^{-/-}$ (44) and IL-12p35$^{-/-}$ (45) mice is on the surface parodoxical as T cells are described as being the main producer of IFN-γ during chronic *Brucella* infections (44, 45) and both MyD88 (44) and IL-12p35 (45) have been implicated in IFN-γ induction. When comparing the CFU counts in organs of IL-12p35$^{-/-}$, CD3ε$^{-/-}$ and IFN-γR$^{-/-}$ mice 35d after i.n. infection (Figure 6.B), we observed that the numbers of brucellae in these organs are similar in CD3ε$^{-/-}$ and IFN-γR$^{-/-}$ mice, but that only the IFN-γR$^{-/-}$ mice died (Figure 6.A). This result suggests that lack of control of bacterial growth is not the only reason underlying the IFN-γ deficiency induced mortality in response to *Brucella* infection.

In order to address this enigma, we analyzed *in situ* by electron and fluorescent microscopy the tissues of wild type, IL-12p35$^{-/-}$, CD3ε$^{-/-}$ and IFN-γR$^{-/-}$ 35d after i.n. infection. We observed that only IFN-γR$^{-/-}$ infected mice displayed major alteration in lung (Figure 7.A), spleen and liver structure (data not shown) at 35d p.i.. These alterations seem mainly due to a dramatic recruitment of Gr1^{+} cells (Figure 7.B for lung and supplementary Figure supplementary S3 for lung, spleen and liver). Flow cytometry analysis identified Gr1^{+} cells in the spleen as neutrophils as demonstrated by their typical cell surface phenotype: Ly-6G^{+} F4/80^{-} MHC-II^{-} CD11b^{+} (supplementary Figure S4). This dramatic neutrophilia is not correlated with a higher frequency of IL-17^{+} cells in IFN-γR$^{-/-}$ infected mice (supplementary Figure S5), suggesting that a compensatory Th17 response developing in the absence of a Th1 response is not implicated in mortality. Interestingly, neutrophilia strongly affects the cellular composition of the granuloma in the liver (Figure 8.A) and spleen (data not shown). We have previously shown (40) that granulomas induced by *Brucella* infection in immunocompent mice are mainly composed of iNOS^{+} monocytes (CD11b^{+} CD11c^{-} F4/80^{low}) and inflammatory dendritic cells (CD11b^{+} CD11c^{+} F4/80^{high}) with few neutrophils (GR1^{+}). We observed a similar cellular composition of granulomas in the liver after i.n. infection in wild type and IL-12p35$^{-/-}$ mice with only a partial reduction of iNOS staining in IL-12p35$^{-/-}$ mice. In contrast,

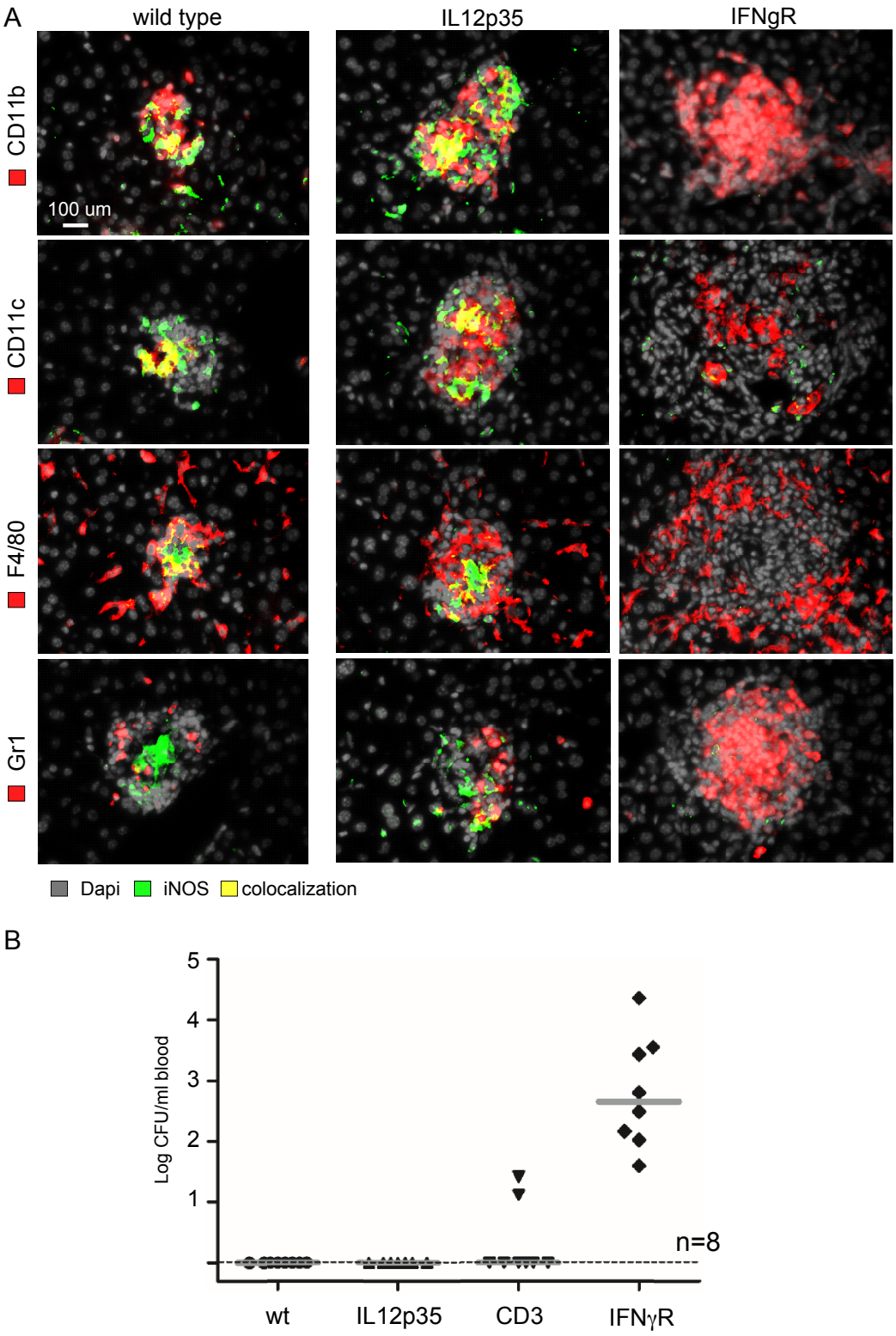

Figure 8: Comparison between wt, IL12p35- CD3- and IFNγR-deficient mice 35 days post infection. Mice were infected i.n. with $2x10^4$ CFU of *B. melitensis* and sacrificed 35 days later. **A,** Phenotypic analysis of granuloma in the liver of infected wt, ILp35- and IFNγR-deficient mice. Panels are color-coded with the text for Dapi or the antigen examined. scale bar=100 µm as indicated. **B,** The data represent the number of CFU per mL of blood in wt, ILp35-, CD3- and IFNγR-deficient mice. Grey bars represent the medians. n denotes the number of mice used for each lineage. Data are representative of at least two independent experiments.

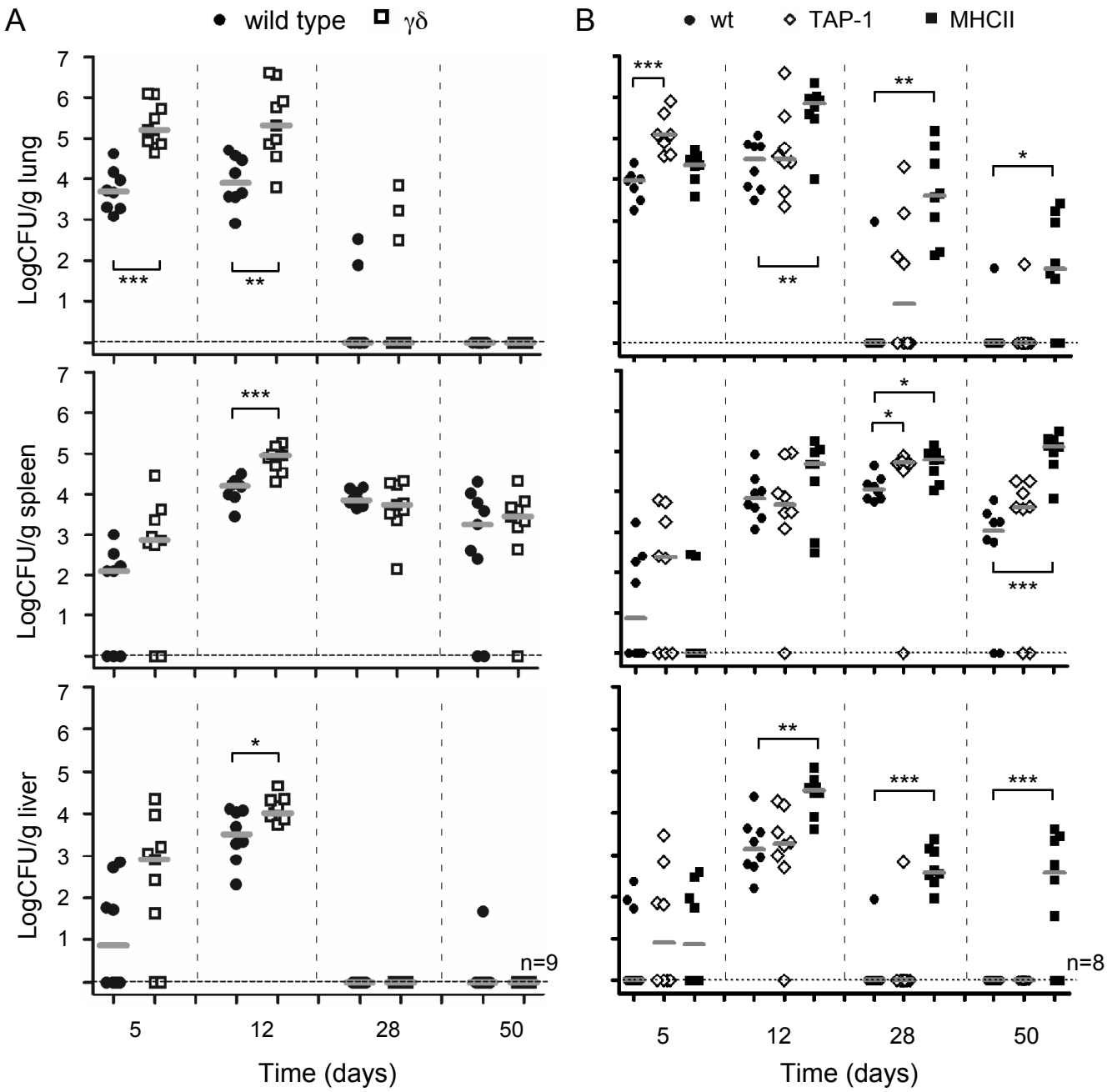

Figure 9: Course of *B. melitensis* mcherry in organs of C57BL/6 wt and deficient mice for different T cell populations. Mice were i.n. infected with a dose of $2x10^4$ CFU of *B. melitensis* and sacrificed at the indicated times. **A,** Comparison between wt and γδ T cell-deficient mice. **B,** Comparison between wt, TAP-1- and MHCII-deficient mice. The data represent the number of CFU per gram of lungs (up), spleen (middle) and liver (down). Grey bars represent the medians. n denotes the number of mice used for each lineage at each time. These results are representative of at minimum two independant experiments. *, P<0,05; **, P<0,01; ***, P<0,001.

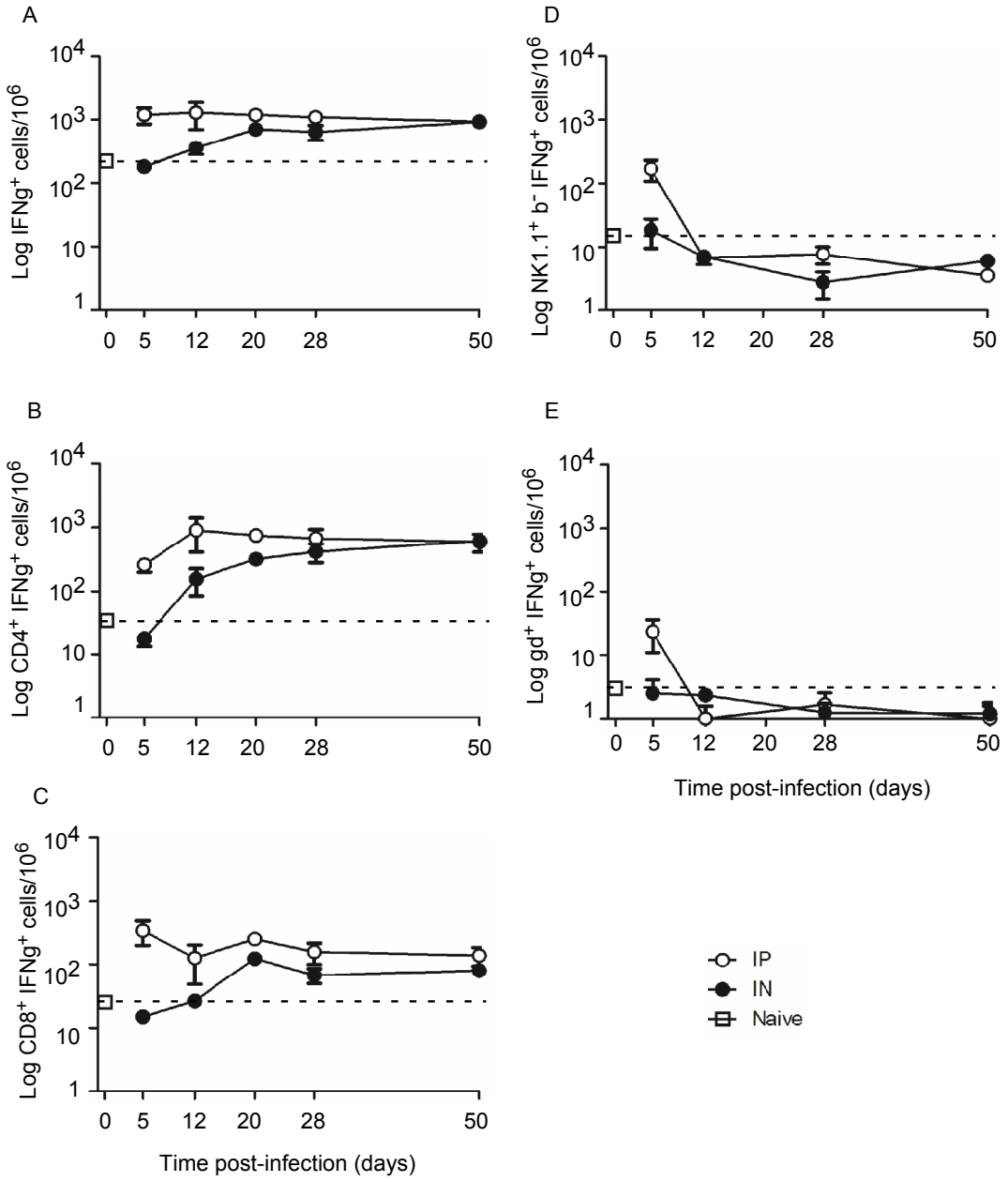

Figure 10: IFNγ production in the spleen of C57BL/6 wt mice after a *B. melitensis* mcherry infection. Mice were i.n. or i.p. infected with a dose of $2x10^4$ CFU of *B. melitensis* and sacrificed at the indicated times. Spleen cells were collected and analyzed by flow cytometry. Cells were gated according to the size and scatter to exclude dead cells and debris for analysis. **A,** Spleen cells were analyzed for total IFNγ production. **B,** Spleen cells were analyzed for CD4 marker versus IFNγ production. **C,** Spleen cells were analyzed for CD8 marker versus IFNγ production. **D,** Spleen cells were analyzed for γδ marker versus IFNγ production. **E,** Spleen cells were analyzed for NK.1.1 and TCRβ markers versus IFNγ production. The data represent the mean ± SEM of the number of IFNγ producing cells **(A)**, $CD4^+$ $IFN\gamma^+$ cells **(B)**, $CD8^+$ $IFN\gamma^+$ cells (**C**), $\gamma\delta^+$ $IFN\gamma^+$ cells **(D)** or $NK1.1^+$ $TCR\beta^-$ $IFN\gamma^+$ cells **(E)** per 10^5 spleen cells acquired at different times after the infection. Square and intermittent line indicate the level of IFNγ production by each cell type in naïve mice. These results are representative of at minimum two independent experiments.

IFN-γR$^{-/-}$ infected mice display large granulomas dominated by iNOS^{-} CD11b^{+} Gr1^{+} CD11c^{-} F4/80^{-} cells (Figure 8.A). These neutrophil-rich granulomas do not seem able to contain *Brucella* infection as they are correlated with large numbers of brucellae in the blood (Figure 8.B). Taken together, these results suggest that IFN-γ signaling deficiency is characterized by a dramatic neutrophilia leading to multiple organ failure and a major alteration of granuloma formation associated with persistence of brucellae in the blood.

γδ T cells and CD4^{+} T cells are the main effector T cell populations controlling *Brucella* growth after primary intranasal infection

In order to determine which lymphocyte populations are implicated in the control of i.n. *Brucella* infection, we compared the course of infection in various strains of C57BL/6 mice genetically deficient for key genes affecting specific lymphocyte subsets.

Absence of γδ T lymphocytes led to a strong defect in early *Brucella* control in the lungs before 12 days p.i (Figure 9.A). γδ T cell deficiency has only a transient impact on colonization of the spleen and liver. In contrast, MHC-II deficiency affecting CD4^{+} T lymphocytes abrogates *Brucella* control in all organs at 12 days p.i. until 50 days p.i., confirming their crucial role in the protective immune response against chronic *Brucella* infections (Figure 9.B). TAP-1 deficiency affecting CD8^{+} T lymphocytes had only minor consequences on the course of infection (Figure 9.B). Thus, while γδ T cells play a role as a first line of defense in mucosal lung tissue, CD4^{+} T cells are involved in the later control of infection.

Comparison of the cell surface phenotypes of IFN-γ producing cells in the spleen following i.p. and i.n. *Brucella* infection showed that CD4^{+} T lymphocytes are the dominant IFN-γ-producing cells at all times during infection (Figure 10). In contrast to i.p. infection, we did not observe an early peak of IFN-γ-producing γδ^{+} T lymphocytes, natural killer or CD8^{+} T

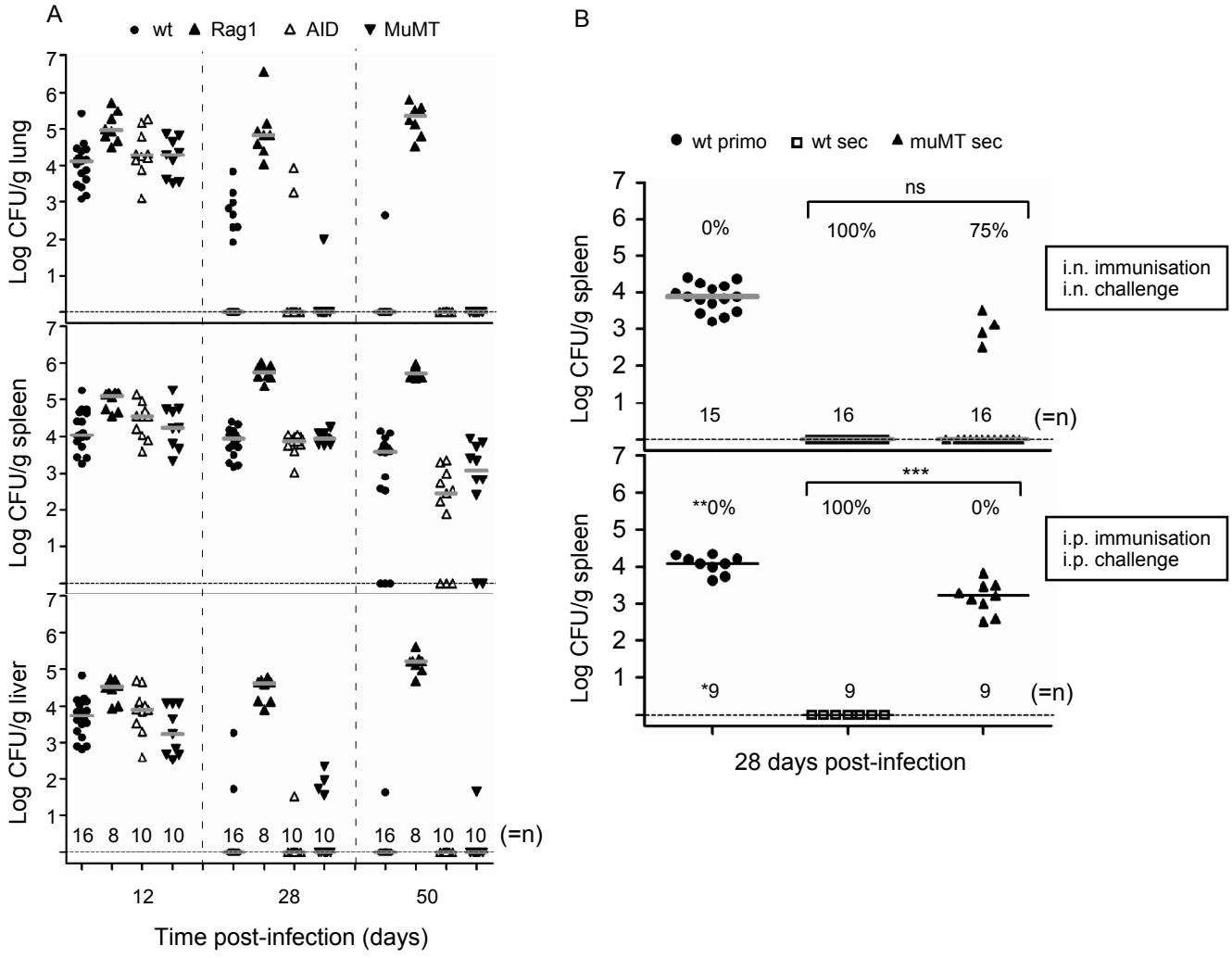

Figure 11: Analysis of the importance of immunoglobulins in the control of *B. melitensis* infection of C57BL/6 mice. A, primary infection. Course of *B. melitensis* mCherry in organs of C7BL/6 wt, Rag1-, AID- and MuMT-deficient mice. They were i.n. infected with a dose of $2x10^4$ CFU of *B. melitensis* and sacrified at the indicated times. The data represent the number of CFU per gram of lungs (up), spleen (middle) and liver (down). Grey bars represent the medians. n represents the number of mice used for each lineage at each time. **B,** secondary infection. **B,** CFU counting of the *B. melitensis* challenge strain in the spleen of C57BL/6 wt and muMT mice. C57BL/6 wt and muMT mice were first intranasally (up) or intraperitoneally (down) infected with a dose of $2x10^4$ CFU of *B. melitensis* (immunization). 50 days later, they were challenged intranasally (up) or intraperitoneally (down) with the same dose of *B. melitensis* mcherry strain (challenge). Immunized mice with *B. melitensis* mcherry strain were used as the control (primo wt). Mice were finally sacrificed 28 days post-challenge. The data represent the number of CFU per gram of spleen. Grey bars represent the medians. n denotes the number of mice used in each group. Two asterisks (**) denote the percentage of mice free of bacteria in the spleen, three asterisks (***) denote significant differences with $P<0,001$, ns denote a non-significant difference. Results are representative of at minimum two independent experiments.

lymphocytes during i.n. infection suggesting that early activation of these cells during i.p. infection is an artifact due to the rapid dissemination of *Brucella* to the spleen.

Humoral immune response plays only a minor role in the control of secondary intranasal *Brucella* infection in mice

Previous studies employing the i.p. *Brucella* infection model from our group (41, 45) and others (49) have shown that B cell deficiency has positive effect on the control of primary infection, but impaired the elimination of *Brucella* from the spleen following a secondary infection.

i.n. *Brucella* infection induced a detectable humoral immune response in the blood (Supplementary Figure S6.A), but we failed to detect a significant *Brucella*-specific antibody response in a bronchoalveolar lavage (data not shown).

The course of *Brucella* infection appears similar in the lungs, spleen and liver of i.n. infected wild type, $AID^{-/-}$ (deficient for activation-induced deaminase-mediated isotype switch) and $MuMT^{-/-}$ (deficient for B lymphocytes) C57BL/6 mice at all times tested (Figure 11.A).

In order to estimate the importance of the humoral immune response in the control of secondary i.n. infection, wild type and $MuMT^{-/-}$ mice that had been infected for 50d with B. melitensis 16M were challenged with an mCherry-expressing derivative of this strain. i.p. challenge was used as an internal control in this experiment. Figure 11.B displays the number of brucellae detected in the spleens at 28d post challenge with the mCherry-expressing *Brucella* strain. We observed that, as previously published (41), the control of i.p. challenge required B lymphocytes as none of the $MuMT^{-/-}$ mice appeared to be able to eliminate the brucellae from the spleen. In striking contrast, 75% of the $MuMT^{-/-}$ mice cleared *Brucella* from this organ after the i.n. challenge. The minor role of B lymphocytes in the control of i.n. challenge is not a consequence of the inability of i.n. infection-induced specific antibodies to recognize *Brucella*. This is demonstrated by the fact that both i.p. and i.n. infected mice are

equally able to control the bacterial level in the blood after i.p. challenge with $5x10^7$ brucellae (Supplementary Figure 6.B).

DISCUSSION

The major entry point for many human pathogens occurs at gastrointestinal, respiratory, or genital mucosal surfaces (reviewed in (16)). Protective immunity against mucosal pathogens will require novel vaccine strategies to induce mucosal immune responses tailored to the anatomic location and the threat of the invading pathogen. However, the requirement for mucosal immunization to generate protective "frontline immunity" against pathogens remains largely undefined. Immune responses required for mucosal protection can differ vastly depending on the individual pathogen. In addition, the route of vaccination appears important for selected and programmed protective immune responses. Despite recent progress in the understanding of *Brucella* infection very few studies (18–23) have investigated the dynamics of infection in several compartments following mucosal lung infection. Here, we compared the location of *B. melitensis* in tissues and the nature of protective immune responses following i.n. infection in mice to our previous results obtained using the classical i.p. infection model (40–42, 45).

In contrast to i.p. infection, i.n. infection does not lead to a rapid dissemination of *Brucella* to the spleen and liver nor to persistence of these bacteria in the blood. This could explain why *Brucella*-induced granulomas in the spleen appeared immediately localized in the white pulp and not in the red pulp as previously described following i.p. infection (40). We hypothesize that i.p. infection saturates the marginal zone filter of the spleen containing marginal zone macrophages, allowing the infection of red pulp F4/80^{+} macrophages. Thus, we demonstrated in our model that the route of infection can alter the tissue localization of the brucellae and the type of host cells these bacteria infect.

The role of humoral immunity in the control of primary and secondary infection by *Brucella* strains appears complex. Our work (45) and that of others (49) has shown that the absence of B cells does not impair the control of primary i.p. infection by *Brucella*. B cell

deficiency can even favor *Brucella* clearance as B cells can be infected by *Brucella* (50) and thus contribute to chronic bacterial infections by providing an intracellular niche that may exert an immunoregulatory role by the production of IL-10 and TGF-β (49). As expected, our results showed that absence of B cells does not affect the course of *Brucella* infection following intranasal infection. However, we have also observed that circulating antibodies play a crucial role in the control of secondary i.p. infection by *Brucella* (41). In the absence of B cells, the Th1 cellular immune response is unable to clear *Brucella* from the spleen after a challenge infection. Following i.p. inoculation, *Brucella* are found associated with erythrocytes in the blood (42). After a brief extracellular phase, *Brucella* is able to invade erythrocytes and to persist for several weeks in the bloodstream. Circulating specific antibodies are able to neutralize *Brucella* before erythrocyte invasion and thus reduce *Brucella* dissemination to organs. In striking contrast to the i.p. infection model, we observed that i.n. challenge is weakly affected by B cell deficiency. We hypothesize that *Brucella* disseminate from the lungs to the spleen and liver inside myeloid phagocytic cells and thus avoid neutralizing antibodies. A result in accordance with this observation is that *Brucella* is rapidly found in dendritic cells and alveolar macrophages in lung draining lymph nodes (43). These results suggest that humoral immunity is dispensable for controlling natural mucosal infection by *Brucella* and that cellular immunity is the key marker of protective immunity.

Studies performed with the i.p. infectious model have clearly established the crucial role of IFN-γ-secreting $CD4^+$ Th1 cells in the control of *Brucella* infections (40, 45–48). Here, we demonstrated that the mortality associated with IFN-γ deficiency is not primarily linked to uncontrolled *Brucella* growth in tissues, but mainly to the strong neutrophilia causing multiple organ failure. Unfortunately, we failed to reduce *Brucella*-induced mortality in IFN-$\gamma R^{-/-}$ mice by repeated administration of neutrophile-depleting antibodies, such as 1A8 or GR1 (data not shown). This could be because the chronic nature of *Brucella* infections leads to continuous recruitment of new neutrophils. This neutrophilia is not associated with a

compensatory Th17 response as we did not detect a higher frequency of IL-17 producing cells in the spleens of infected IFN-γR$^{-/-}$ mice when compared to infected wild type mice. In agreement, cutaneous and musculoskeletal inflammation observed following i.p. *Brucella* infection in IFN-γ$^{-/-}$ mice is independent of the Th17 response (51). In the *Ehrlichia muris* infection model, it has been shown (52) that IFN-γ acts to control infection by directly promoting the differentiation of myeloid cells toward monocytes. In the absence of IFN-γ signaling, infection promotes exclusively the expansion of neutrophils. These latter cells do not play a protective role during *Brucella* infection (53) and are generally associated with massive tissue damage, particularly in the lungs (reviewed in (54)). In agreement, in our model, absence of IFN-γ signaling is associated with drastic alteration of the granuloma composition that appeared mainly composed of neutrophils and lack of tissue confinement of *Brucella* as demonstrated by the high level of bacteria observed in the blood of IFN-γR$^{-/-}$ mice during chronic but not the early phase of infection. In conclusion, our results strongly suggest that the protective role of IFN-γ against *Brucella* infection has been over interpreted based on the exceptional mortality in mice associated with IFN- γ deficiency (46, 48). IFN-γ is clearly indispensable for *Brucella* control but is not sufficient as we have observed (45) that a similar frequency of IFN-γ-producing CD8$^+$ T cells does not replace IFN-γ-producing CD4$^+$ T cells to control *Brucella* growth in the spleen.

The Th17 response has been mainly associated with host defense against several extracellular pathogens (reviewed in (55)). However, a growing number of studies have also associated Th17 with the control of intracellular microorganisms, such as *Listeria monocytogenes*, *Salmonella enterica*, and *Mycobacterium tuberculosis* (reviewed in (56)). Up to now, studies of the function of Th17 responses in immunity against *Brucella* infections have been scarce. In the classical i.p. infection model, we have reported that IL-23p19 deficiency does not impact the control of *Brucella* in the spleen, confirming that Th1 and not Th17 plays a major role in the clearance of brucellae from this organ. In the present work, we

observed similar results in the spleen after i.n. infection. Both IL-17RA and Il-23p19 deficiency have only a minor or no effect on CFU counts in the spleen and liver at all times post infection. In striking contrast, IL-17RA$^{-/-}$ mice display a limited capacity to control Brucella infections in the lungs. This defect is limited to the early stages of infection, e.g. prior to 12 days p.i.. Interestingly, during this period, IL-23p19 and IFN-γR deficiency have no, or minor, effect on the CFU counts of *Brucella* in the lungs. These results suggest that the Th17 response constitutes the first line of defense against *Brucella* infections in the lungs. The Th1 response develops later, and in the absence of IL-12p35, the Th1 response can be replaced in the lungs by a Th17 response, as demonstrated by the fact that wild type and IL-12p35$^{-/-}$ mice display similar levels of control at later times during the infection. A similar compensatory phenomenon is also observed in the liver, suggesting that the key role of IL-12-induced Th1 in the spleen may be the exception and not the general rule. The Th17 response is generally considered to be strictly dependent on IL-23, but reduced IL-17 production can be detected even in IL-23$^{-/-}$ mice (30). Unfortunately, we failed to identify the source of IL-17 in the lungs (data not shown). However, we observed that γδ T cell deficiency, but not CD4^{+} T cell deficiency, also impacts early *Brucella* control in the lungs, suggesting that γδ T cells could be a potential source of IL-17 in our model.

In summary, our work highlights the importance of identifying protective immune responses in more realistic experimental models. We showed that bacterial localization in organs and effector immune mechanisms required to control infection are strongly dependent on the route of infection. In the intranasal infectious model, we identified CD4^{+} T cell-mediated Th1 and Th17 cellular immune responses as the core protective immune responses against *Brucella* and exclude a major role for B cells and CD8+ T cells. These findings could improve our ability to develop protective vaccines or therapeutic treatments against brucellosis. As CD4+ T cell dependent cellular immunity only developed following the administration of live bacteria (41), live attenuated vaccines, or inactivated yet metabolically

active, seem to remain the easiest and most potent tools for the production of candidate protective vaccines against brucellosis.

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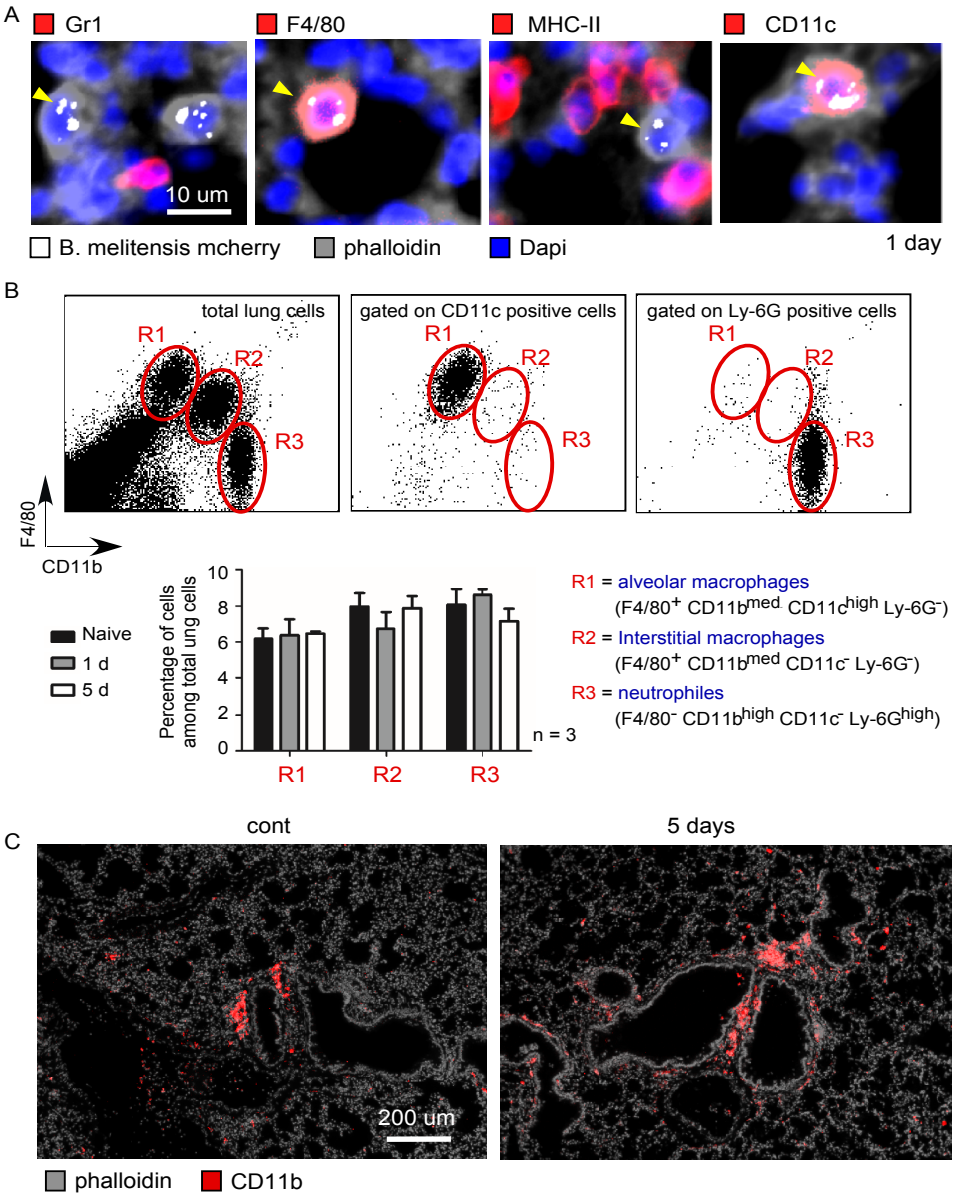
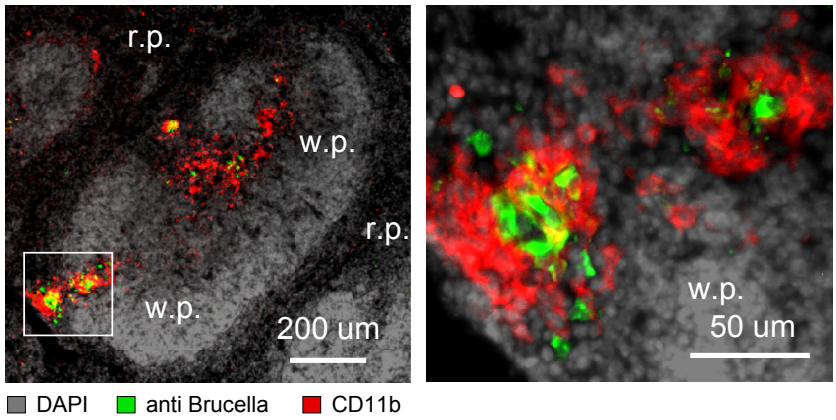
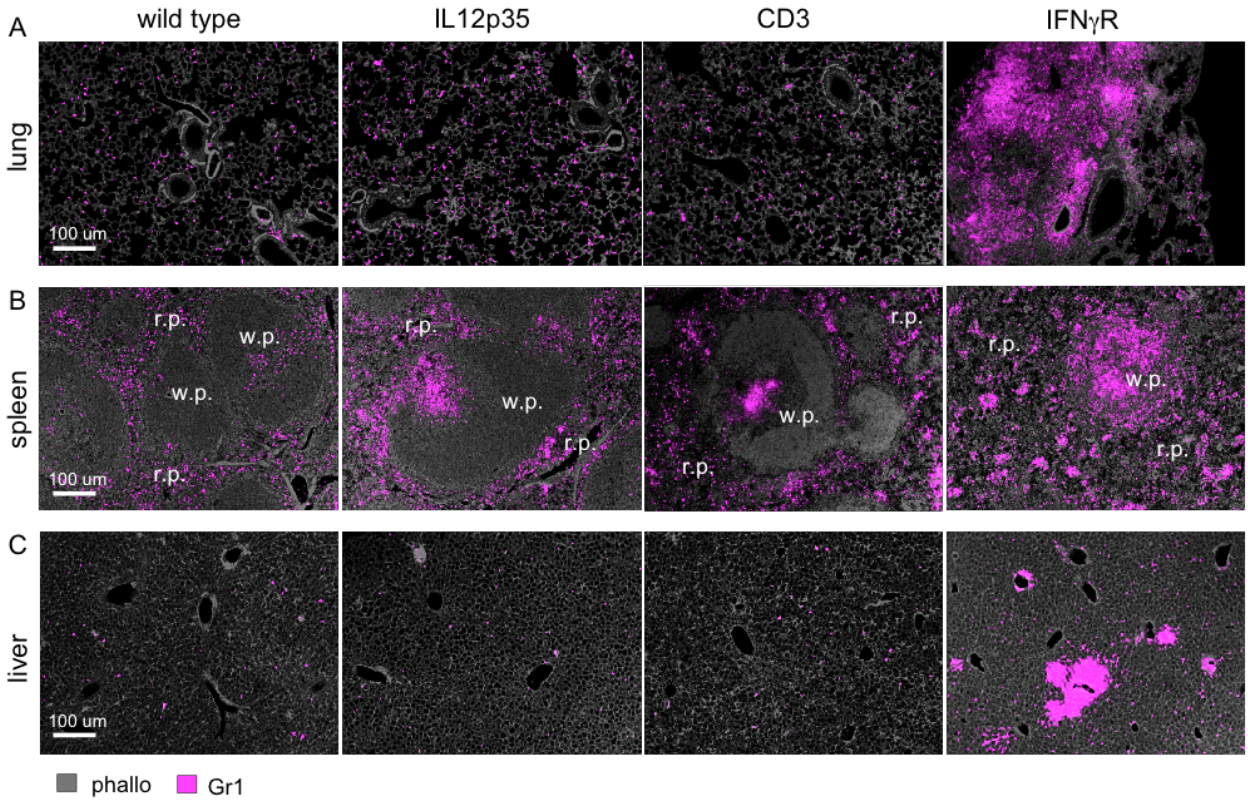


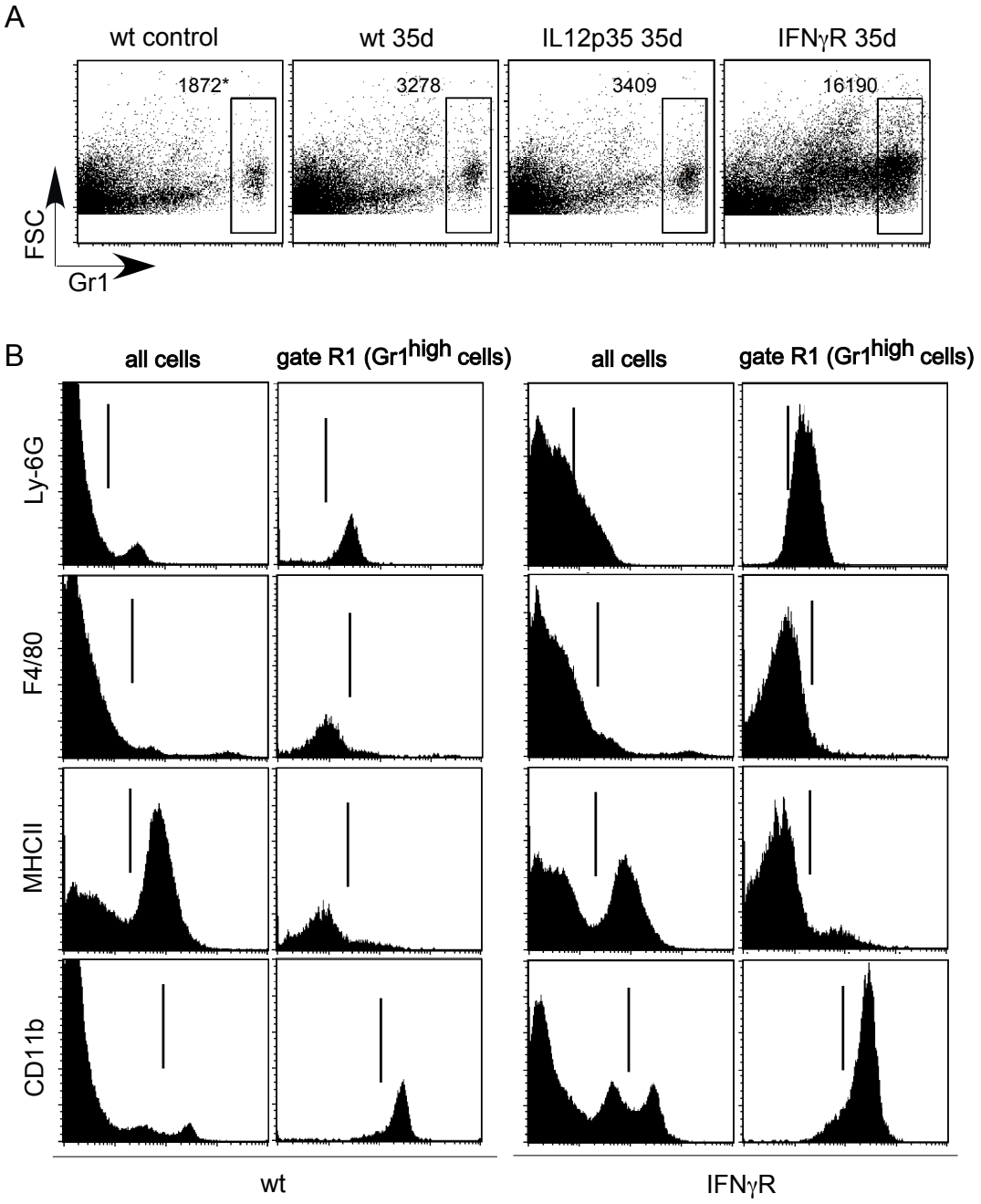
S1: analysis of lungs in C57BL/6 wt mice. Mice were infected i.n. with a dose of $2x10^4$ CFU of *B. melitensis* and sacrified at the indicated times. Naïve mice were used as a control (cont). **A,** lungs were collected and examined by immunohistofluorescence. The panels represent the phenotype of infected cells in lungs 1 day post-infection. Panels are color-coded with the text for Dapi, phalloidin, mCherry-Bru or the antigen examined. Yellow arrowheads indicate the presence of bacteria. **B,** lungs cells were collected and analyzed by flow cytometry. Cells were gated according to the size and scatter to exclude dead cells and debris for analysis. lung cells were analyzed for F4/80, CD11b, Ly6G and CD11c markers. The panels shows representative dot plots from an individual lung. The left figure shows total lung cells analyzed for the F4/80 marker versus CD11b marker per 10^5 cells acquired, the middle figure shows lung cells gated on CD11c positive cells (R1) and the right figure shows lung cells gated on Ly6G positive cells (R3). The graph indicates the mean ± SEM of the percentage of cells in gate R1 (alveolar macrophages as indicated), R2 (interstitial macrophages as indicated) and R3 (neutrophiles as indicated) per 10^5 cells acquired in naïve mice, 1 day (d) and 5 days infected mice. n denotes the number of mice used. **C,** lungs were collected and examined by immunohistofluorescence. The panels represent a section of a naive lung (left) and a lung 5 day post-infection (right). Panels are color-coded with the text for phalloidin and the antigen examined (CD11b). . Scale bar= 10 and 200 µm. These results are representative of at minimum two independent experiments.



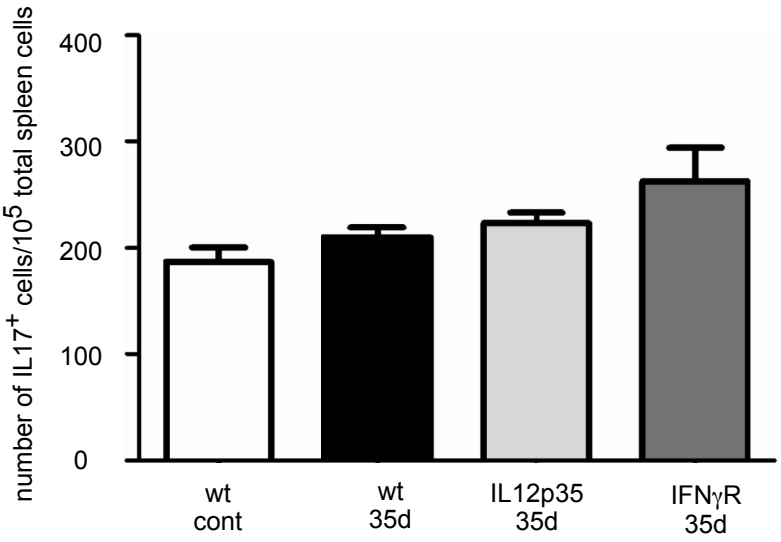
S2: localization of *Brucella* in the spleen of C57BL/6 wt mice. Mice were infected i.n. with a dose of $2x10^4$ CFU of *B. melitensis* and sacrified at 20 days post infection. The left figure shows the localization by immunofluorescence of CD11b-expressing cells and cells stained with anti-*Brucella* Ab in spleen. The right figure is higher magnification view of the left one. Panel is color-coded with the text for Dapi, anti-*Brucella* or the antigen examined (CD11b). Scale bar=200 µm and 50 µm, as indicated. r.p.: red pulp; w.p.: white pulp. Data are representative of at least two independent experiments.



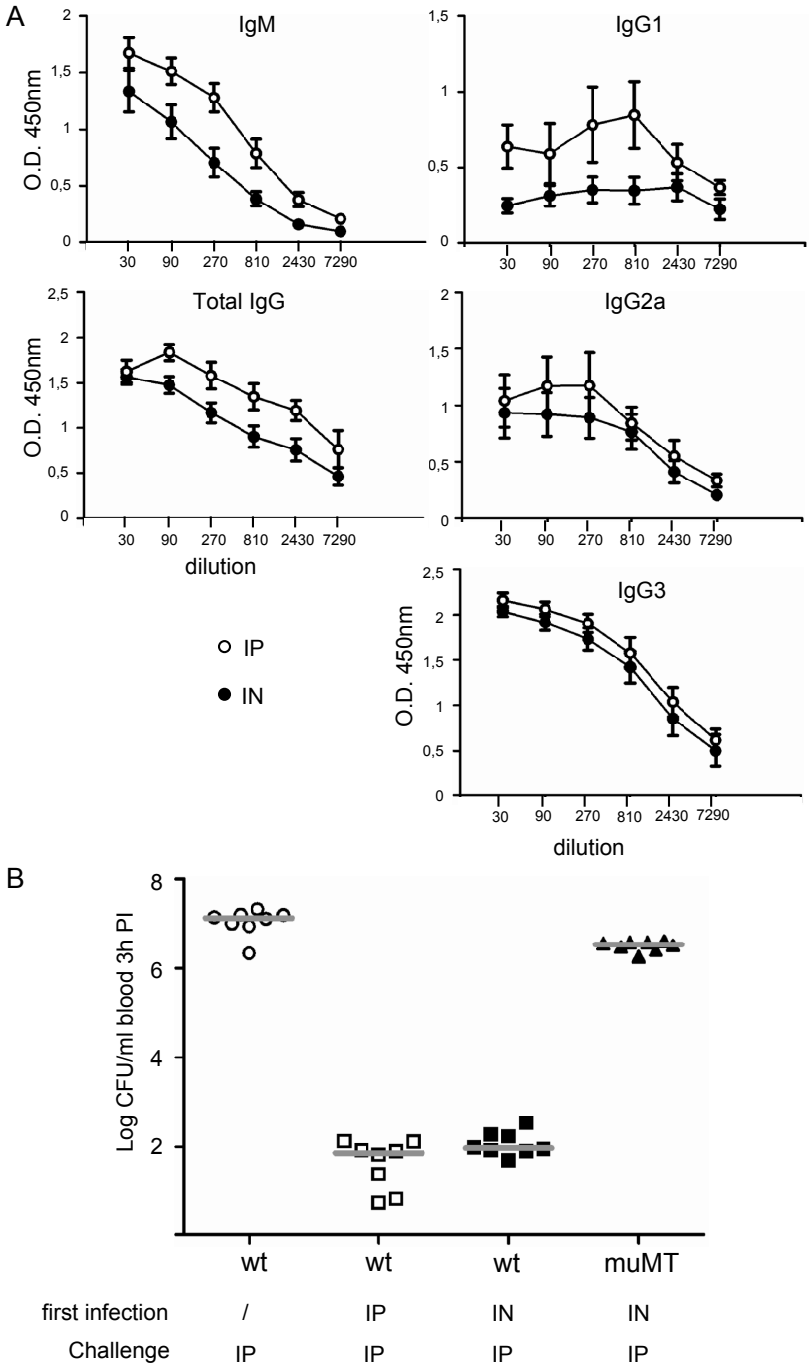
S3: Study of Gr1-expressing cells in lungs, spleen and liver of C57BL/6 wt, IL12p35-, CD3- and IFNγR- deficient mice. Mice were infected i.n. with a dose of $2x10^4$ CFU of *B. melitensis* and sacrified 35 days post infection. Organs were collected and examined by immunohistofluorescence. The panels show Gr1-expressing cells in lungs **(A)**, spleen **(B)** and liver **(C)** of wt, IL12p35-, CD3- and IFNγR- deficient mice from the left to the right. Panels are color-coded with the text for phalloidin and the antigen examined (Gr1). Scale bar= 100 µm. as indicated. r.p.: red pulp; w.p.: white pulp. These results are representative of at minimum two independent experiments.



S4: Phenotyping of Gr1^{high} cells into the spleen of C57BL/6 WT, IL12p35 and IFNγR-deficient mice. Mice were i.n. injected with 2x10^4 CFU of *B. melitensis* and killed at indicated times. Spleen cells were collected and analysed by flow cytometry. Cells were gated according to the size and scatter to exclude dead cells and debris for analysis. **A,** Spleen cells were analysed for Forward Size Scatter (FSC) versus Gr1 production. The figure shows representative dot plots from individual spleen in each group. One asterisk (*) denotes the number of cells in gate R1 out of 10^5 cells acquired. **B,** Gr1 ^{high} spleen cells (R1) were selected for wt and IFNγR-deficient mice and analyzed for the expression of a panel of markers: Ly6G, F4/80, MHCII and CD11b. The vertical line delimits the Gr1 ^{high} cells. The results are representatives of at minimum two independent experiments.



S5: Cytokine IL-17A production in the spleen of C57BL/6 wt, IL12p35 and IFNγR-deficient mice. Mice were i.n. injected with $2x10^4$ CFU of *B. melitensis* and killed 35 days post-infection. Naïve wt mice were used as a control (cont). Spleen cells were collected and analyzed by flow cytometry. Cells were gated according to the size and scatter to exclude dead cells and debris for analysis. Spleen cells were analyzed for Forward Size Scatter (FSC) and IL17A production. The data represent the mean ± SEM of the number of IL17A positive cells per 10^6 spleen cells acquired. The results are representatives of at minimum two independent experiments.



S6: Comparison of *B. melitensis* specific Immunoglobulins and CFU in the blood of i.p. versus i.n. infected C57BL/6 wt and muMT-deficient mice. A, wt mice were i.p. or i.n. infected with a dose of $2x10^4$ CFU of *B. melitensis* and sera were collected 50 days later. The data represent the mean ± SEM of the optical density that increases with the presence of immunoglobulins in the serum. 6 dilutions have been analyzed. **B,** wt and muMT mice were immunized with a dose of $2x10^4$ CFU of *B. melitensis*. One group of wt mice was i.p. immunized and one group of wt and muMT mice was i.n. immunized. 50 days later, all mice were i.p. infected a second time (challenge) with *B. melitensis* mcherry strain. I.p. immunized mice with *B. melitensis* mcherry strain were used as a control. Blood was drawn 3 hours post infection. The data represent the number of CFU per mL of blood 3 hours post-infection. Grey bars represent the medians. n denotes the number of mice used for each lineage. These results are representative of at minimum two independent experiments.

Research is creating new knowledge
Neil Armstrong

2. The phenotype of Brucella melitensis infected cells in vivo is independent of STAT6

Delphine Hanot Mambres, Carl De Trez, Jean-Jacques Letesson,, Eric Muraille

This is a preliminary manuscript describing the results obtained during the second part of research described in this thesis. It does not correspond to the final version of a published paper. We would like to associate these results with the ones obtained by Arnaud Machelard and Georges Potemberg working on the combination of asthma and *Brucella* infection in mice. Their work will complete our study by showing the impact of an induced Th2 immune response in wild-type mice on *Brucella* infection and reservoir cells.

It has been demonstrated that alveolar macrophages are the first cells to be infected when aerosolized *Brucella* colonize the lungs of mice (Archambaud *et al*., 2010). It is also known that trophoblasts are a preferential location during pregnancy in natural hosts (Andreson et al., 1986), and that *Brucella* is able to infect a large panel of cells, including professional phagocytes (Billard *et al.*, 2005; Copin *et al.*, 2012). However, the range of host cells colonized during the chronic phase of the disease is unknown. Research in mice has shown that that the extremities, joints in the tail and the spleen are anatomic locations where persistent *Brucella* infections can be found (Rajashekara *et al.,* 2005; Vitry *et al*., 2012).

The objective of this part of the work was to analyze infected cells in the spleens of mice during the "chronic" phase of infection. Unfortunately, studying reservoir cells in the spleens of wild-type mice is particularly complicated because of the limited number of infected cells during this phase of the infection. Thus, we decided to use susceptible IL12p40-deficient BALB/c mice that show higher CFU counts in the spleen at 28 days post-infection. This mouse lineage does not develop an effective Th1 immune response but has a pro-Th2 bias. A recent study has suggested that M2 macrophages, developed during a Th2 immune response, could be the preferential niche for *Brucella* during chronic infection (Xavier *et al.*, 2013). We decided to test this hypothesis by depletion of the STAT6 gene in IL12p40-deficient mice. These double knockout mice do not develop an effective Th1/Th17 immune response and do not Th2-dependent M2 macrophages.

Interestingly, bacterial numbers and the phenotypes of reservoir cells were not influenced by STAT6 pathway. This suggests that infected cells are not Th2-induced M2 macrophages, even if they are positive for the M2 marker Arg1. The staining profiles of infected cells ($CD11c^+$, $DEC205^+$, $Arg1^+$ and high lipid content) reveals characteristics shared by foamy macrophages encountered during *Mycobacterium tuberculosis* infection. Thus, *Brucella* may manipulate the host environment to produce a favorable niche full of nutrients for its replication and persistence like *Mycobacterium tuberculosis*.

The phenotype of *Brucella melitensis* infected cells *in vivo* is independent of the STAT6 dependent signaling pathway.

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Running title: Phenotype of *Brucella* infected cells

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ABSTRACT

Brucella are zoonotic bacterial pathogens that establish long-term intracellular infections in their host. Recent studies suggest that chronic phase of *Brucella* infection is associated with induction of alternatively activated (M2) macrophages. In several infectious models, it has been shown that M2 macrophages are induced by IL-4/IL-13 (Th2) cytokines and can constitute a favorable niche for long-term bacterial persistence. In this study, we analyzed *in situ* the phenotype of *Brucella melitensis* infected cells in the spleen from highly susceptible IL-12p40$^{-/-}$ BALB/c mice and the impact of STAT6 deficiency on this phenotype. Surprisingly, we observed that the absence of IL-4/IL-13 signaling pathways in IL-12p40$^{-/-}$ STAT6$^{-/-}$ mice does not affect the bacterial number in the spleen nor the phenotype of reservoir cells when compared to IL-12p40$^{-/-}$ mice. In both mouse strains, the phenotype of infected spleen cells appears heterogeneous and mainly comprised of dendritic cells (CD11c^{+}) or marginal zone macrophages (MOMA1^{+} and ERTR9^{+}) expressing low levels of MHC-II and located in the T cell area of the white pulp. These cells frequently express Arginase1 but are negative for Fizz1 or CD301 M2 markers. They also contain high level of lipid droplets detected by bodipy staining suggesting the presence of high-energy carbon sources in these cells. Taken together, we formally demonstrated that formation of reservoir cells in susceptible mice is independent of the STAT6 signaling pathway and as a consequence that the phenotype of *Brucella*-infected cells is not dependent on Th2 cytokines. This characterization of *Brucella* reservoir cells could help to better understand *Brucella* persistence in the host and define more efficient therapeutic strategies.

INTRODUCTION

A growing number of clinically relevant infectious diseases are characterized by pathogen persistence in the host. Chronic and recurrent infections require long-lasting and costly therapy and are the cause of much morbidity in the world [1]. Brucellosis, considered one of the most common global zoonoses, affects a large range of mammals and is caused by facultative intracellular Gram-negative bacteria of the genus *Brucella* [2,3]. Human brucellosis is a severe and debilitating disease that may lead to permanent damage and requires prolonged and combined antibiotic therapy [4]. Direct cutaneous contact, ingestion of animal products and inhalation of airborne agents are the main routes of transmission in humans [5]. No safe or effective vaccine is available to prevent human brucellosis [6].

Very little is known about the phenotype and the physiological state of the host cells that harbor *Brucella* strains during chronic infections. Following intraperitoneal [7] or intranasal infection [8], the spleen is chronically colonized by *Brucella*. Using a *B. melitensis* 16M strain expressing the mCherry fluorescent protein (mCherry-Br), we previously observed [9] in wild type C57BL/6 mice the formation of $iNOS^+$ granuloma structures surrounding infected spleen cells. Inflammatory dendritic cells ($iNOS^+$ $CD11b^+$ $CD11c^+$ $F4/80^+$ $MHC\text{-}II^+$) and activated monocytes ($iNOS^+$ $CD11b^+$ $CD11c^-$ $F4/80^+$ $MHC\text{-}II^+$) appeared as the major infected cell types in the granulomatous lesions. Alteration of the MyD88/IL-12/IFN-γ axis dramatically affects the cellular composition of these granulomas, reducing their ability to control bacterial dissemination and leading to replication and persistence of the *Brucella* in distinct cellular niches. In the spleen of highly susceptible IL-$12p40^{-/-}$ BALB/c mice, these cells are located in the T cell area of the white pulp, express CD11c, MOMA-1 and DEC-205 C-type lectin (CD205) markers and are negative for iNOS, CD11b and F4/80 markers associated to microbicidal activity [9].

The Th2 cytokines IL-4 and IL-13 induce, through STAT6 dependent signaling, the polarization of macrophages toward an alternatively activated (also termed M2) phenotype. These later cells are characterized by the selective expression of M2 markers such as Arginase1 (Arg1), Fizz1 (Found in Inflammatory Zone 1) [10] and MGCL1 (Macrophage Galactose-C-type lectin, CD301) [11], and display low microbicidal activity and a lipid oxidative metabolism (reviewed in [12–14]). In various infectious models, it has been shown that M2 macrophages can constitute a favorable niche for long term persistence of bacteria [15,16] and protozoa [17,18]. A recent report [19] suggests that the chronic phase of *Brucella* infection is associated with the induction of M2 macrophages and that these cells could constitute a favorable intracellular niche for *Brucella* persistence. In this study, we analyzed *in situ* the phenotype of *Brucella melitensis* infected cells in the spleens from highly susceptible IL-12p40$^{-/-}$ BALB/c mice and the impact of STAT6 deficiency on this phenotype. Surprisingly, we observed that the absence of IL-4/IL-13 signaling pathways in IL-12p40$^{-/-}$ STAT6$^{-/-}$ mice does not affect the phenotype of *Brucella*-infected cells when compared to IL-12p40$^{-/-}$ mice. This demonstrates that the formation of reservoir cells in susceptible mice is independent of STAT6 signaling pathways, and consequently that these cells are not Th2-induced M2 cells even if they express some M2 markers such as Arginase1.

MATERIALS AND METHODS

Ethics Statement

Procedures of this study and mice handling is conform with current European legislation (directive 86/609/EEC) and the corresponding Belgian law “Arrêté royal relatif à la protection des animaux d'expérience du 6 avril 2010 publié le 14 mai 2010". The Animal Welfare Committee of the Université de Namur (UNamur, Belgium) has reviewed and approved the complete protocol (Permit Number: 05-558).

Mice and reagents

Wild-type BALB/c mice were acquired from Harlan (Bicester, UK). We used also STAT6$^{-/-}$ BALB/c mice (strain C.129S2-*STAT6*^{tm1Gru}/J), IL12p40$^{-/-}$ BALB/c mice (C.129S1-*IL12b*^{tm1Jm}/J), both purchased from Jackson Laboratory, and the double knockout STAT6/IL12p40 BALB/c mice, obtained by cross between the two simple knockout cited above. All wild-type and deficient mice used in this study were bred in the animal facility of the Gosselies campus of the Université Libre de Bruxelles (ULB, Belgium).

We used a strain of *Brucella melitensis* 16M stably expressing a rapidly maturing variant of the red fluorescent protein DsRed (Shaner et al. 2004): the mCherry protein (mCherry-Br), under the control of the strong *Brucella* spp. promoter, PsojA. Construction of the mCherry-Br strain has been described previously in detail (Copin et al. 2012). It grows in biosafety level III laboratory facilities. Cultures grew overnight with shaking at 37 °C in 2YT media (Luria-Bertani broth with double quantity of yeast extract) and were washed twice in RPMI 1640 (Gibco Laboratories) (3500xg, 10 min.) before the inoculation in mice.

Mice infection

Mice were anaesthetized with a cocktail of Xylasin (9mg/kg) and Ketamin (36 mg/kg) in PBS before being inoculated intra-nasally (i.n.) with $2x10^4$ CFU of wild type or mCherry-expressing *B. melitensis* in 30 µL of RPMI. Control animals were inoculated with the same volume of RPMI. Plating serial dilutions of inoculums validated the infectious doses. At the selected time after infection, mice were sacrificed by cervical dislocation. Immediately after sacrifice, spleen, liver and lung cells were collected for bacterial count, flow cytometry and/or microscopic analyses.

Bacterial count

Spleens, livers and lungs were crushed and transferred in PBS/0.1% X-100 triton (Sigma). We performed successive serial dilutions in RPMI to get the most accurate bacterial count and plated them on 2YT medium. The CFU were counted after 5 days of culture at 37°C.

Immunofluorescence microscopy

Spleens were fixed for 4 hours at room temperature in 2% paraformaldehyde (pH 7.4), washed in PBS, and incubated overnight at 4°C in a 20% PBS-sucrose solution. Lungs were submitted to the same treatments but in a vacuum chamber at room temperature. Tissues were then embedded in the Tissue-Tek OCT compound (Sakura), frozen in liquid nitrogen, and cryostat sections (5 nm) were prepared. For the staining, tissue sections were rehydrated in PBS and incubated in a PBS solution containing 1% blocking reagent (Boeringer) (PBS-BR 1%) for 20 minutes before being incubated overnight in a PBS-BR 1% containing mAbs or reagents: DAPI nucleic acid stain Alexa Fluor 350 or 488 phalloidin (Molecular Probes) to visualize the structure of the organ, and Allophycocyanin (APC)-coupled BM8 (anti-F4/80, Abcam), Alexa Fluor 647-coupled M1/70 (anti-CD11b, BD Biosciences), Alexa Fluor 647-coupled HL3 (anti-CD11c, BD biosciences), Alexa Fluor 647-coupled 53-6.7 (anti-CD8α, Santa Cruz technology), Alexa Fluor 647-coupled RB6-8C5 (anti-Gr1, ebioscience), APC-coupled M5/114.15.2 (anti-MHCII, I-A/I-E), Alexa Fluor 647-coupled NLDC-145 (anti-Dec205/CD205, Biolegend), Alexa Fluor 647-coupled ER-MP23 (anti-CD301, AbD Serotec), Bodipy 493/503 (Molecular Probes), Biotin-coupled HL3 (anti-CD11c, BD Biosciences) Biotin-coupled RB6-8C5 (anti-Gr1, ebioscience), Biotin-coupled MOMA1 (anti-Marginal Zone Macrophages, BMA Biomedicals), Biotin-coupled ERTR9 (anti-SIGN-R1, BMA Biomedicals), Biotin-coupled HL3 (anti-CD11c BD Biosciences), IgG H-52 (anti-Arg1, Santa Cruz technology), and IgG Fizz (anti-RELMα, Abcam) to stain the cells of interest. Incubation with a streptavidin coupled fluorochrome for 2 hours was necessary for the biotin-coupled Ab: Alexa Fluor 350 or Alexa Fluor 647 streptavidin (Molecular Probes). Incubation with a secondary antibody Alexa Fluor 647-coupled goat anti-rabbit IgG (Molecular Probes) was necessary for the anti-Arg1 and anti-Fizz mAb. Slides were mounted in Fluoro-Gel medium (Electron Microscopy Sciences, Hatfield, PA). Labelled tissue sections were visualized with an Axiovert M200 inverted microscope (Zeiss, Iena, Germany) equipped with a high-resolution

monochrome camera (AxioCam HR, Zeiss). Images (1384x1036 pixels, 0.16µm/pixel) were acquired sequentially for each fluorochrome with A-Plan 10x/0.25 N.A. and LD-Plan-NeoFluar 63x/0.75 N.A. dry objectives and recorded as eight-bit grey-level *.zvi files. At least 3 slides per organ were analyzed from 3 different animals and the results are representatives of 2 independent experiments.

Statistical analysis

We used a (Wilcoxon-) Mann-Whitney test provided by GraphPad Prism software to statistically analyze our results. Each group of deficient mice was compared to wild-type mice. We also compared each group with each other and displayed the results when required. Values of $p < 0.05$ were considered to represent a significant difference and were noted by *.

DNA extraction, Polymerase Chain Reaction (PCR) and electrophoresis gel to define mice lineages

DNA extraction from tail of mice was carried out with high pure PCR template preparation kit (ROCHE): 0,2 cm of tails were incubated overnight at 55°C with 40 µL of Proteinase K in 200 µL of Tissue lysis buffer. Samples were sheared few seconds with a vortex before adding 200 µL of binding buffer and 100 µL of isopropanol. They were mixed and centrifuged during 5 minutes at 13,000x g. Liquid sample was pipeted in a high filter tube inserted in a collection tube and centrifuged 1 minute at 8,000x g. The filter tube was removed from the collection tube and combined with a new one. 500 µL of inhibitor removal buffer were added to the upper reservoir of the filter tube and all of it was centrifuged at 8,000x g during 1 minute. The collection tube was discarded and the filter tube was combined with a new one before the addition of 500 µL of wash buffer to the upper reservoir of the filter tube and centrifugation was made during 1 minute at 8,000x g. The washing step was made a second time. Then, the filter tube was inserted into a clean microcentrifuge tube. 200 µL of prewarmed (70°C) elution buffer to the upper reservoir of the filter tube. We centrifuged all of it 1 minute at 8,000x g. By this step, the eluted DNA was released in the microcentrifuge tube.

1 µL of Extracted DNA was mixed with 5 µL of Green buffer 5x, 2 µL of desoxyribonucleotides (dntp) 5mM, 1 µL of $MgCl_2$ 25mM, 15.2 µL of H_20, 0,2 µL of GoTaq

polymerase (2.5mM each) and 0,2 µL of each primer (see table 1 below for primer sequences). PCR cycling parameters are as follow: denature at 94°C for 3 minutes, anneal at 66°C for 1 min and extend at 72°C for 1min. 35 cycles were programmed. Finally, PCR products were pipeted on 1% agarose gel in 1x TAE buffer for migration to check their size.

Primers used to amplify STAT6 gene	
primers	***Sequence (5'-3')***
oIMR7416 wt	AGTGGGTCCCCTTCACTCT
oIMR1822 common	CTCCGGAAAGCCTCATCTT
oIMR0092 Neo	ATCCATCTTGTTCAATGGCCGATC

Primers used to amplify IL12p40 gene	
primers	***Sequence (5'-3')***
oIMR0457 wt	AGTGAACCTCACCTGTGACACG
oIMR0458 common	TCTTTGCACCAGCCATGAGC
oIMR6916 Neo	CTTGGGTGGAGAGGCTATTC

RESULTS

STAT6 deficiency does not affect the control of *Brucella* infection in IL-12p40$^{-/-}$ mice

Based on indirect observations, it has been recently proposed that the reservoir cells for *Brucella* in the spleens of experimentally infected mice could be M2 macrophages (19). In order to test this hypothesis, we developed a model by which *Brucella*-infected cells can be visualized *in situ* and where the differentiation of M2 macrophages is favored by the absence of Th1. In a previous study (7), we have shown that IL-12p40$^{-/-}$ BALB/c mice are highly susceptible to *Brucella* infection and display higher bacterial numbers in the spleen during the chronic phase of infection allowing the direct observation of infected cells *in situ* with mCherry-expressing *Brucella melitensis* (mCherry-Br). In these mice, both protective Th1 and Th17 responses were severely impaired by the absence of functional IL-12 and IL-23 complexes. We compared these mice with IL-12p40$^{-/-}$ STAT6$^{-/-}$ BALB/c mice in a model of intranasal infection using inoculums of $2x10^4$ CFU (low dose) or $2x10^7$CFU (high dose) of mCherry-Br. STAT6 deficiency neutralizes the signaling pathways of IL-4R and impairs the formation of M2 macrophages [20][21]. Wild type and STAT6$^{-/-}$ BALB/c mice were used as internal controls. The CFU counts in the lungs, spleens and livers were analyzed at 12, 28 and 50 days post infection (Figure 1). The absence of STAT6 and IL-12p40 functional genes in each group of deficient mice has been confirmed by PCR (supplementary Figure 1). Wild-type mice and STAT6$^{-/-}$ mice display similar CFU counts following inoculation with a low or high infectious dose, in all organs and at all time tested, confirming previous results [7] showing that absence of a Th2 response does not favor a Th1 protective response. Surprisingly, we observed that absence of a functional STAT6 signaling pathway does not affect the control of infection even in absence of a Th1 response in IL-12p40$^{-/-}$ mice. Only a weak and brief difference is observed at 28 days post infection in the lungs following low dose infection. The absence of a STAT6 deficiency impact on the control of *Brucella* infection

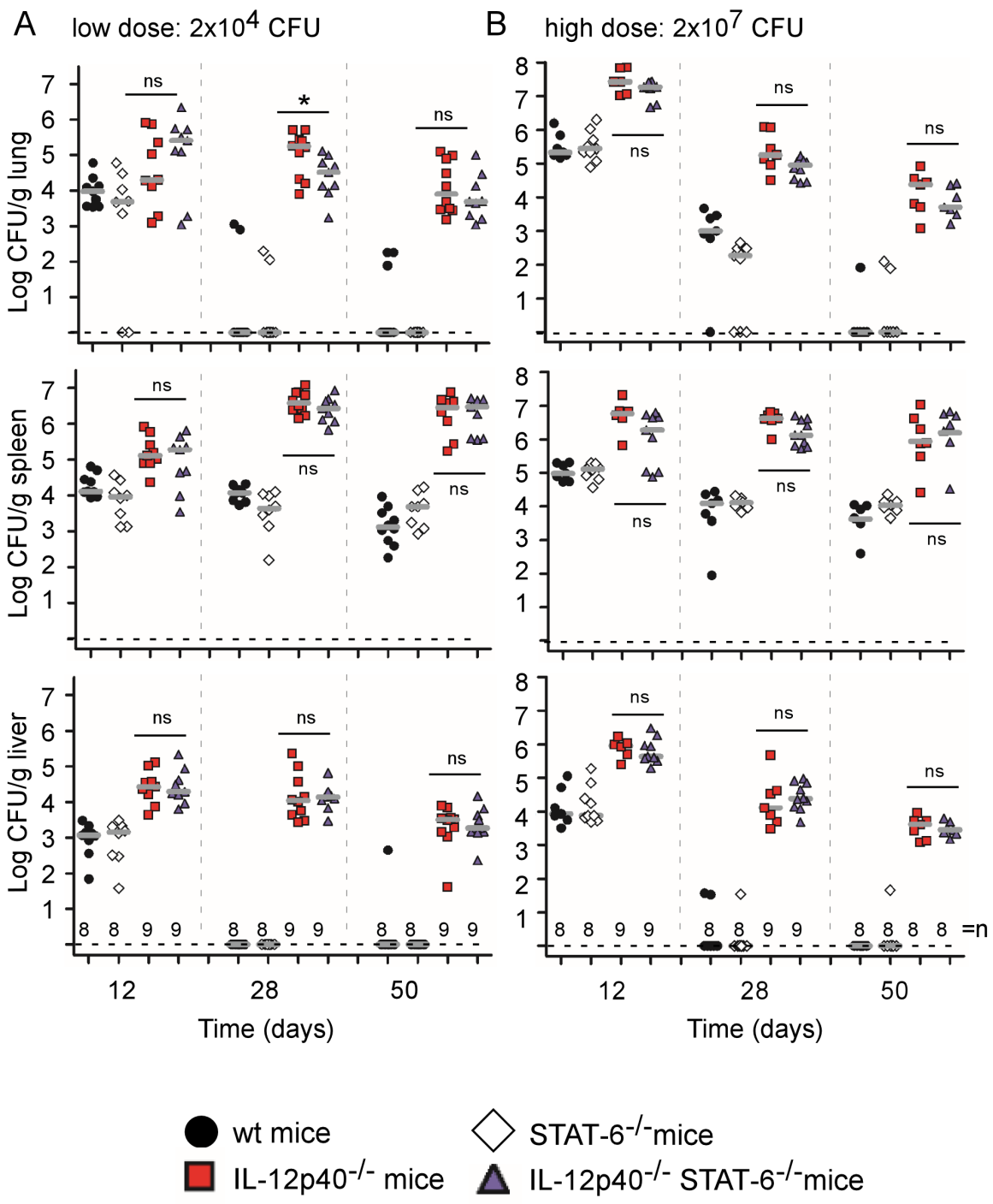

Figure 1: Course of *B. melitensis* infection in organs of BALB/c wild-type (wt), STAT6-, IL12p40- and STAT6/IL12p40-deficient mice. **A,** Mice were intranasally (i.n.) injected with 2.10^4 CFU of *B. melitensis* and sacrificed at the indicated times. **B,** Mice were i.n. injected with 2.10^7 CFU of *B. melitensis* and sacrificed at the indicated times. The data represent the number of CFU per gram of lung, spleen and liver. Grey bars represent the medians. "n" is the number of mice used. These results are representative of at minimum two independent experiments. *, P<0,05; ns, non-significant.

does not support the hypothesis that M2 macrophages constitute a reservoir for the persistence of *Brucella in vivo*.

STAT6 deficiency does not modify the phenotype of *Brucella* infected cells in IL-12p40$^{-/-}$ mice

Direct observation of mCherry-Br *in situ* requires a minimum of 5x10^{6} CFU/g in organs (9). Unfortunately, this limiting condition is only achieved in the spleens of IL-12p40$^{-/-}$ mice (Figure 1). In order to determine the impact of STAT6 deficiency on the phenotype of infected spleen cells, we compared *in situ* using fluorescence microscopy the expression of various cell surface markers on infected cells in the spleens from IL-12p40$^{-/-}$ and IL-12p40$^{-/-}$ STAT6$^{-/-}$ mice. We analyzed the expression of GR1, F4/80, CD11b, CD11c, CD8a, MHC-II, MOMA-1, ER-TR9 on a minimum of 200 infected cells from 3 animals per group and in two different experiments. Signal intensity of expression associated with mCherry *Brucella* was classified as high, low and negative. High corresponds to the highest staining observed in tissue. The frequency of each level on infected cells from both mouse strains is presented in Figure 2. The general distribution for each staining and an example of each signal level is presented Figure 3. No significant difference between STAT6$^{+/+}$ and STAT6$^{-/-}$ mice was observed regardless of the markers tested. Accordingly, we present only staining from IL-12p40$^{-/-}$ mice to illustrate each staining.

As previously reported [9], during the chronic phase of spleen infection infected cells locate mainly in the white pulp of the spleen and preferentially in the T cell area (data not shown).

A recruitment of GR1^{+} cells (presumably neutrophils, based on the characteristic nucleus visualized by DAPI staining) in the red and white pulp is observed in infected mice when compared to naive mice. However, as previously observed [9], only rare infected cells

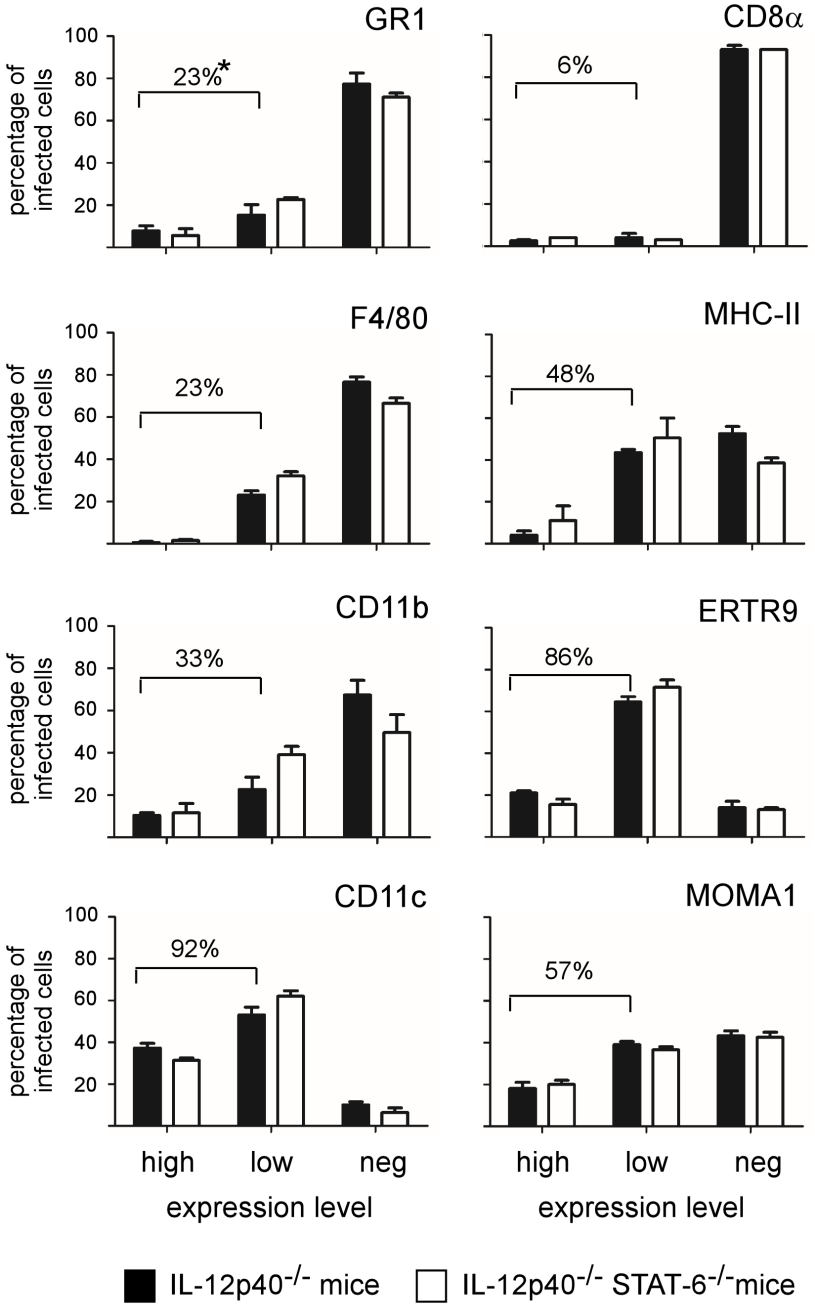

Figure 2: Comparison of the phenotype of infected cells in spleen of BALB/c IL12p40- and IL12p40/STAT6- deficient mice. IL12p40- and IL12p40/STAT6-deficient BALB/c mice were injected i.n. with 2.10^7 CFU of mCherry-Br. Mice were sacrificed at 28 days post-infection and spleens were collected and examined by immunohistofluorescence. Data represent a comparative analysis of the percentage of mcherry-Br that colocalize or not with GR1-, F4/80-, CD11b-, CD11c-, CD8a-, MHCII-, ER-TR9- and MOMA-1- expressing cells (high and low expression) in the two lineages of mice. These results are representative of at minimum three independent experiments. *, Percentage of colocalization between mCherry-Br and positive cells for the antigen (addition of high and low expression) in IL12p40-deficient mice.

colocalize with GR1 staining. Most of them express low GR1 staining and are observed in dense aggregates of cells rending the precise identification of infected cells uncertain.

Following intraperitoneal infection of wild-type mice, the majority of infected cells in granulomas exhibit CD11b, CD11c and F4/80 staining [9], a phenotype corresponding to iNOS-producing inflammatory dendritic cells (DC) [22]. In our intranasal model, only the CD11c marker appears predominantly expressed on infected cells. 92% of these latter cells express high or low levels of CD11c for 33 and 23% of CD11b and F4/80, respectively. As expected, CD11b staining strongly colocalized with GR1 staining, suggesting that a majority of infected $CD11b^+$ cellsare also $GR1^+$ (data not shown) and are neutrophils. The frequency of infected cells expressing high levels of F4/80 is particularly low.

In the spleens of naive mice, CD8a is mainly expressed on T cells and a subset of DC and were located mainly in the T cell area of the white pulp. Very rare infected cells colocalize with CD8a staining (6%) and the majority express low levels, suggesting that $CD11c^+$ infected cells are rarely CD8a positive. Interestingly, MHCII, generally expressed at high levels on splenic DC, is mainly expressed at low levels on infected cells. Numerous $CD11c^+$ infected cells express low or undetectable level of MHCII (supplementary Figure 2), suggesting that *Brucella* infection could reduce the expression of MHC-II on DC *in vivo*.

A strong association between ER-TR9 (SIGN-R1), MOMA-1 markers and infected cells is also observed. In naive mice, these markers are mainly present in the marginal zone and identify marginal zone macrophages (MZM) and metallophilic marginal zone macrophages (MMM), respectively. Surprisingly, colocalization between CD11c and low levels of MOMA-1 (supplementary Figure 3) and ER-TR9 (data not shown) on infected cells is observed. This phenomenon is mainly found in dense aggregates of cells in the white pulp.

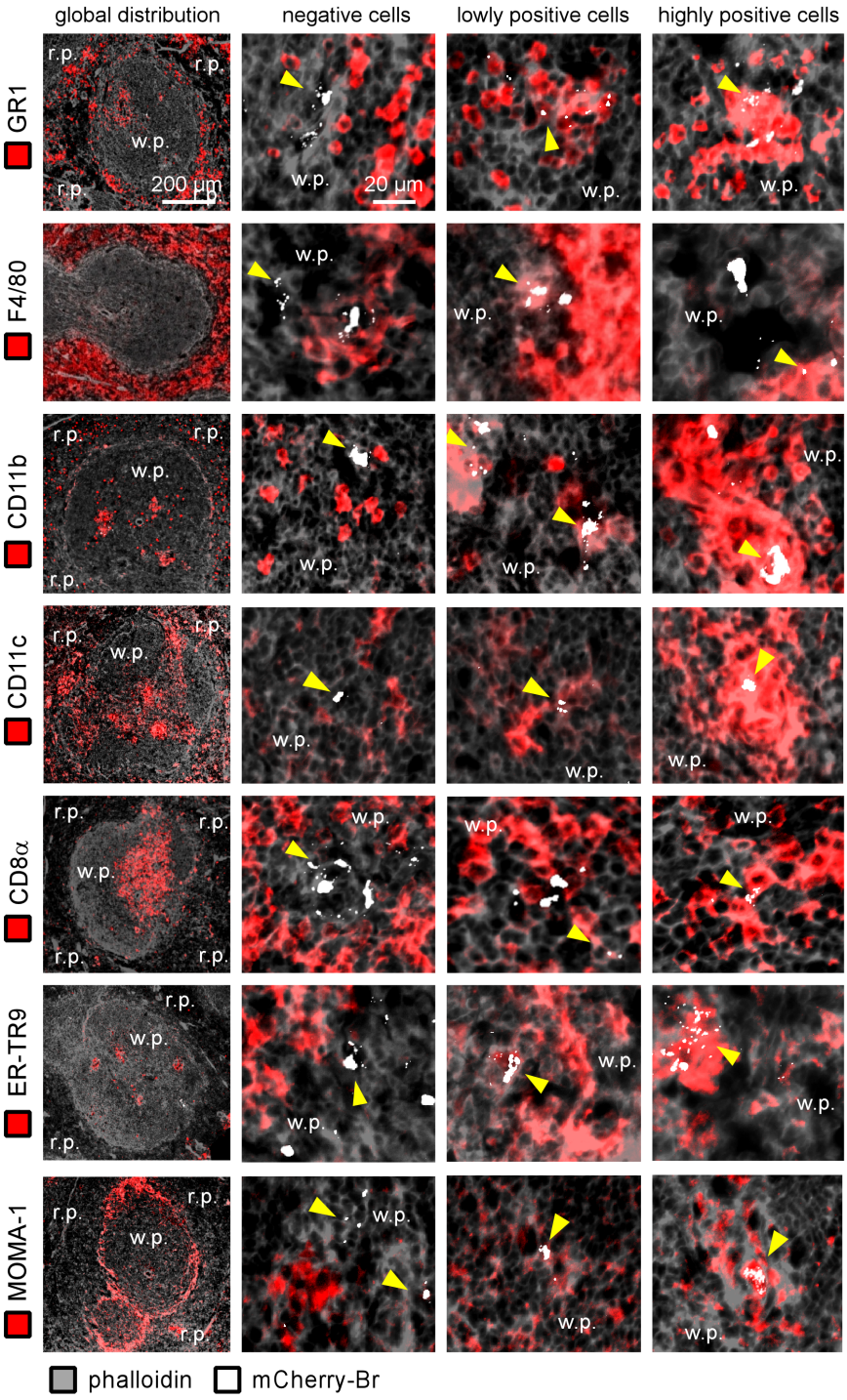

Figure 3: Characterization of infected cells in the spleen of BALB/c IL12p40-deficient mice. IL12p40-deficient BALB/c mice were injected i.n. with 2.10^7 CFU of mCherry-Br. Mice were sacrificed at 28 days post-infection and spleens were collected and examined by immunohistofluorescence. The left panels show global distribution of the Gr1-, F4/80-, CD11b-, CD11c-, CD8a-, ERTR9 and MOMA-1-expressing cells in the spleen. The panels to the right of the first ones show mCherry-Br colocalization with negative cells for Gr1, F4/80, CD11b, CD11c, CD8a, ER-TR9 and MOMA-1. The panels to the right are immunofluorescence analysis of mCherry-Br colocalization with weakly positive cells for Gr1, F4/80, CD11b, CD11c, CD8a, ER-TR9 and MOMA-1. The right panels show mCherry-Br colocalization with highly positive cells Gr1, F4/80, CD11b, CD11c, CD8a, ERTR9 and MOMA-1. Panels are color-coded with the text for phalloidin, the antigen examined or mCherry-Br. Scale bar= 200 and 20 µm, as indicated. r.p.: red pulp:; w.p.: white pulp. Yellow arrowheads indicate the presence of bacteria. Data are representative of at least three independent experiments.

Brucella infected cells do not express typical M2 markers such as Fizz1 and CD301

In order to better characterize the phenotype of infected cells, we analyzed the expression of the DC marker DEC-205 (CD205) and of several well established M2 differentiation markers such as Arginase1 (Arg1), Fizz1 and CD301 expression (Figure 4.A). A strong association of CD205 and Arg1 staining was observed with infected cells. The majority of highly infected cells (displaying large aggregates of mCherry-Br) expressed high levels of both markers (Figure 4.B). In contrast, Fizz1 and CD301 displayed weak colocalization with mCherry-Br signal staining (Figure 4.A) and was mainly located in the red pulp and marginal zone (Figure 4.B). As previously observed for other markers, no significant differences between $STAT6^{+/+}$ and $STAT6^{-/-}$ mice were observed for all markers tested.

As expected, a strong colocalization of CD11c and CD205 on infected cells is observed (Figure 5.A). Similarly, the staining for MOMA-1 (Figure 5.B) and ER-TR9 (data not shown) colocalize with the CD205 staining on infected cells. It is interesting to see that CD205 expression on MOMA-1 cells is mainly found on infected cells but not on MOMA-1+ cells located in the marginal zone (Figure 5.B). Similar results were observed for ER-TR9 (data not shown).

Brucella infected cells are lipid rich cells

Finally, in order to investigate the metabolism of *Brucella* infected cells, we analyzed the level of lipid present in infected cells using Bodipy staining (Figure 6). A very strong colocalization between mCherry-Br and bodipy staining is observed in both strains of mice (Figure 6.A). This colocalization is observed on both isolated and cells within aggregates (Figure 6.B). As expected, Bodipy staining also colocalizes with CD11c (Figure 6.C) and Arg1 (Figure 6.D) staining on infected cells.

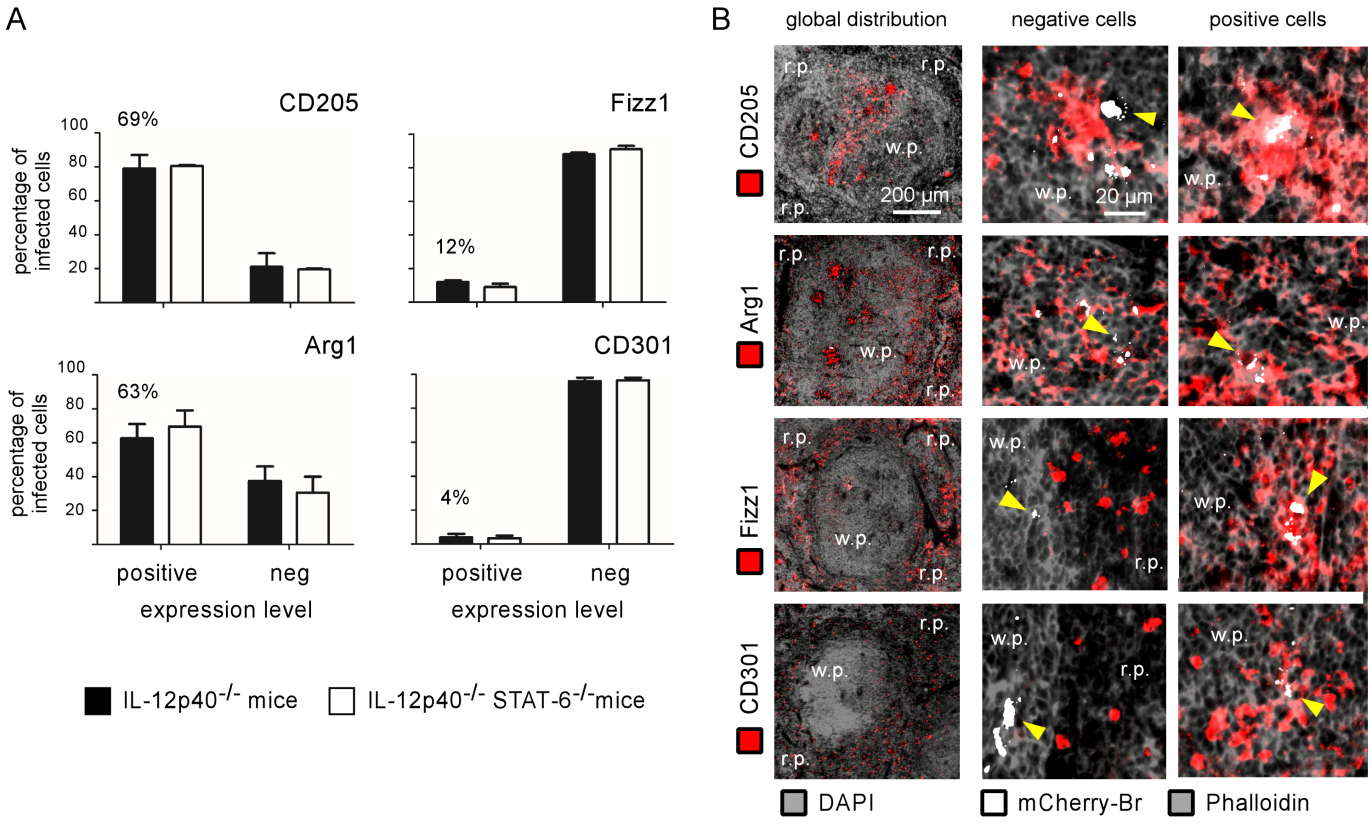

Figure 4: Characterization of infected cells with CD205 expression and M2 markers in the spleen of BALB/c IL12p40- and STAT6/IL12p40-deficient mice. IL12p40-deficient BALB/c mice were injected i.n. with 2.10^7 CFU of mCherry-Br. Mice were sacrificed at 28 days post-infection and spleens were collected and examined by immunohistofluorescence. **A,** Comparative analysis of the percentage of mcherry-Br that colocalize or not with Dec205-, Arg1-, Fizz1- and CD301-expressing cells in spleen of BALB/c IL12p40- and IL12p40/STAT6- deficient mice. The percentage of colocalization between mCherry-Br and positive cells for the antigen in IL12p40-deficient mice is indicated. **B,** Immunohistofluorescence analysis of CD205 and M2 markers in spleen of infected IL12p40-deficient mice and colocalization with mCherry-Br. The left panels show global distribution of the Dec205-, Arg1-, Fizz1- and CD301-expressing cells in the spleen. The middle panels show mCherry-Br colocalization with negative cells for Dec205, Arg1, Fizz1 and CD301. The right panels are immunofluorescence analysis of mCherry-Br colocalization with positive cells for Dec205, Arg1, Fizz1 and CD301. Panels are color-coded with the text for DAPI, phalloidin, the antigen examined or mCherry-Br. Scale bar= 200 and 20 µm, as indicated. r.p.: red pulp:; w.p.: white pulp. Yellow arrowheads indicate the presence of bacteria. Data are representative of at least two independent experiments.

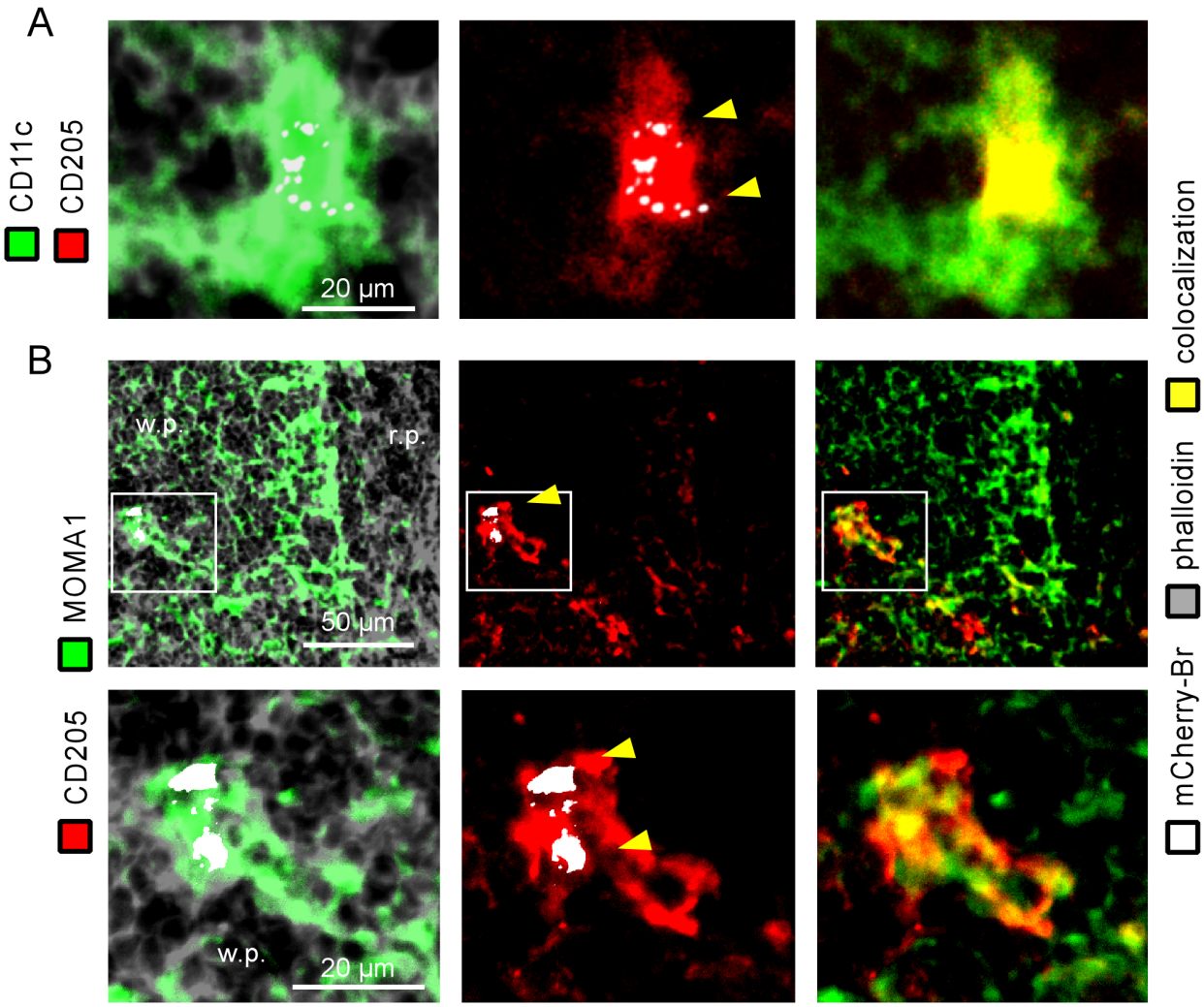

Figure 5: Characterization of infected cells expressing CD11c, CD205 and MOMA-1 in the spleen of BALB/c IL12p40-deficient mice. IL12p40-deficient BALB/c mice were injected i.n. with 2.10^7 CFU of mCherry-Br. Mice were sacrificed at 28 days post-infection and spleens were collected and examined by immunohistofluorescence. **A,** The left panels show mCherry-Br colocalization with cells expressing CD11c. The middle panels show mCherry-Br colocalization with cells expressing CD205 and the right panels show colocalization of CD11c- and CD205-expressing cells. **B,** The upper panels show distribution of MOMA-1-expressing cells and colocalization with mCherry-Br (left), distribution of DEC205-expressing cells and colocalization with mCherry-Br (middle), and colocalization of MOMA-1- and CD205-expressing cells (right). The panels below are higher magnification views of the same stainings. Panels are color-coded with the text for phalloidin, the antigen examined or mCherry-Br. Scale bar= 50 and 20 µm, as indicated. r.p. : red pulp. w.p.: white pulp. Yellow arrowheads indicate the presence of bacteria. Data are representative of at least two independent experiments.

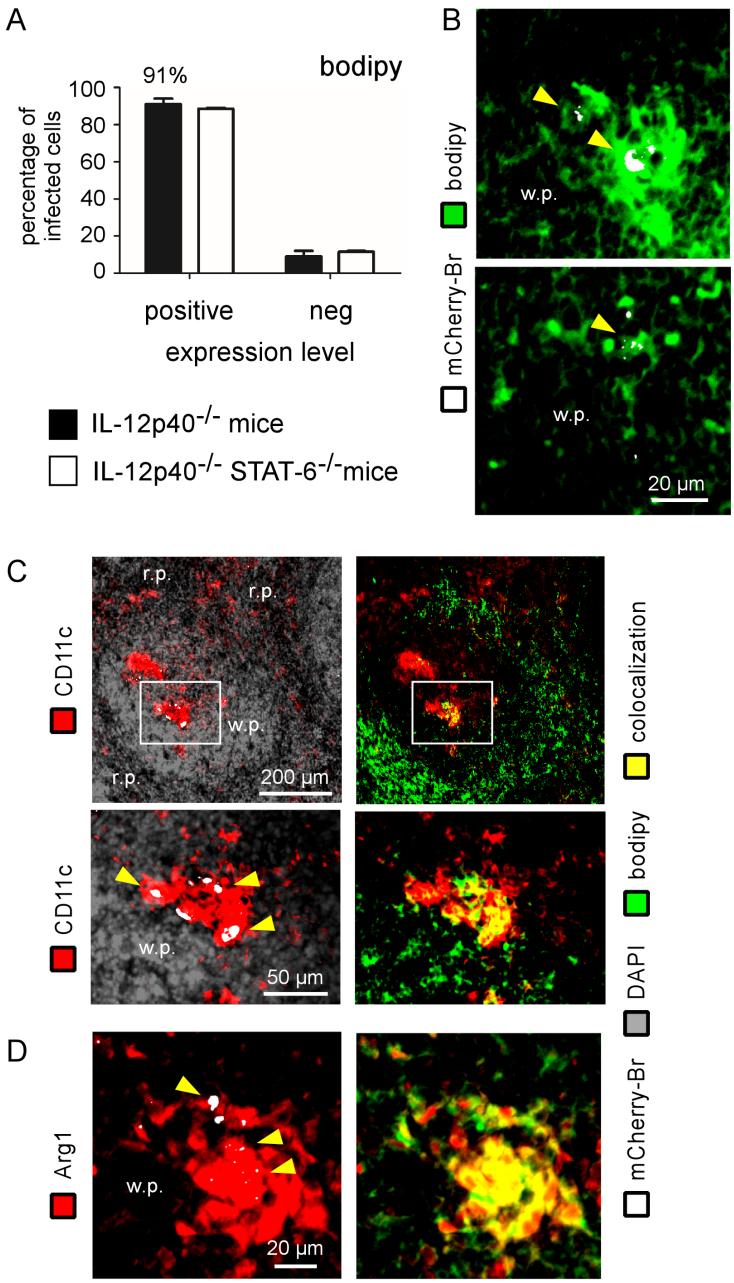

Figure 6: Characterization of the lipid metabolism of infected cells and Arg1 expression in the spleen of BALB/c IL12p40-deficient mice. IL12p40-deficient BALB/c mice were injected i.n. with 2.10^7 CFU of mCherry-Br. Mice were sacrificed at 28 days post-infection and spleens were collected and examined by immunohistofluorescence. **A,** Data represent a comparative analysis of the percentage of mcherry-Br that colocalize or not with cells stained with Bodipy. The percentage of colocalization between mCherry-Br and cells stained with Bodipy in IL12p40-deficient mice is indicated. **B,** Colocalization of mCherry-Br and cells stained with Bodipy. **C,** The upper panels show distribution of CD11c-expressing cells and colocalization with mCherry-Br (left), and distribution and colocalization of Bodipy staining and CD11c-expressing cells (right). The panels below are higher magnification views of the same stainings. **D,** The left picture shows colocalization between Arg1-expressing cells and mCherry-Br, the right picture shows colocalization between Arg1-expressing cells and Bodipy staining. Panels are color-coded with the text for DAPI, the antigen examined, Bodipy or mCherry-Br. Scale bar= 200, 50 and 20 µm, as indicated. r.p. : red pulp. w.p.: white pulp. Yellow arrowheads indicate the presence of bacteria. Data are representative of at least two independent experiments.

DISCUSSION

Recently acquired evidence suggests that persisting pathogens are able to reprogram host cellular microenvironments to their advantage. They frequently polarize the phenotype of host cells to produce an anti-inflammatory and nutriment rich environment assuring their long term persistence in the host (reviewed in [12–15]). According to this new paradigm, it appears crucial to identify the main reservoir cells of bacteria during chronic infection and to define the signaling pathways implicated in their polarization during infectious processes.

Taken together, our analysis of the phenotypes of *Brucella*-infected cells in highly susceptible IL-12p40-/- BALB/c mice confirms previous observations obtained using the intraperitoneal infection model [9] which showed that these cells express mainly CD11c (92%) and CD205 (69%) receptors, two well defined markers of DCs. The absence of CD11b and F4/80 staining demonstrates that these cells are distinct from $CD11b^+$ $CD11c^+$ $F4/80^+$ infected inflammatory DCs observed during the acute phase of infection in wild-type mice [9]. The absence of CD8a expression showed that they are also distinct from the $CD11b^-$ $CD11c^+$ $CD8a^+$ $DEC205^+$ classical DC subset located in the T cell area of naive mice [23]. Coexpression of MOMA-1 and SIGN-R1/ER-TR9 on a fraction of CD11c infected cells suggests that some of these cells could be from the marginal zone. Like DCs, both $MOMA\text{-}1^+$ MMM and $ER\text{-}TR9^+$ MZM migrate from the marginal zone to the T cell area in response to *Listeria monocytogenes* infection [24]. Initially exclusively considered to be macrophages, MZM have recently been divided into $MHC\text{-}II^-$ and $MHCII^+$ cells. The latter display a high capacity to ingest and present antigens to T cells [25] during *L. monocytogenes* infection. In our *Brucella* model, the low level of MHC-II observed on infected cells suggests that $ER\text{-}TR9^+$ infected cells are "real" MZMs infected the in marginal zone that have migrated to the T cell area. To our knowledge, co-expression of MOMA-1 and ER-TR9 with CD11c has not been described and could be a consequence of infection and/or migration.

Recent work [19] has shown that M2 macrophages (identified by CD301 and Fizz1 expression *in situ*) are induced during the chronic phase of *Brucella abortus* infection in wild type C57BL/6 mice. Based on the observation that M2 macrophages are strongly infected *in vitro* and that PPAR agonists, known to induce M2 macrophages polarization [26], can affect the course of infection, the authors concluded that M2 macrophages can be a preferential niche for *Brucella in vivo*. In the majority of infectious and non-infectious experimental models [20,21], M2 macrophages are dependent on the STAT6 signaling axis for their induction. In our model, we found that intranasal *B. melitensis* infection of IL-12p40$^{-/-}$ BALB/c mice induces a recruitment of M2 macrophages to the spleen, but that *Brucella*-infected cells do not express typical M2 markers such as Fizz1 and CD301, and that the course of infection or the phenotype of infected cells is not affected by STAT6 deficiency. Taken together, these results strongly suggest that, even if infected cells express high level of Arg1, they should not be considered as "classical" M2 macrophages, which are dependent upon the Th2 response and the STAT6 signaling pathway. These results, together with our previous report [7] showing that the absence of a Th2 response does not favor *Brucella* elimination from BALB/c mice, suggest that the Th2 response plays only a minor role in the persistence of *Brucella* in the host.

During the chronic phase of infection, the phenotype of the major type of *Brucella*-infected spleen cell (CD11c^{+} CD205^{+} bodipy^{+} Arg1^{+}) is strongly reminiscent of the phenotype displayed by the 'foamy cells' observed in the lungs during *Mycobacterium tuberculosis* infection. These cells also express both CD11c and CD205 markers, low levels of MHC-II [27], and high levels of lipid and Arg1 [28,29]. High levels of lipid droplets in infected cells is induced by several intracellular pathogens such as *M. tuberculosis*, *M. leprae*, *Chlamydia* [31], and *Toxoplasma* (reviewed in [32]) suggesting that these pathogens induce the accumulation of lipids to use them as carbon and energy sources. This has led in

investigators to consider foamy macrophages as nutrient rich reservoir cells that facilitate bacterial persistence *in vivo* [30]. Interestingly, it has recently been shown in the *M. tuberculosis* model that lipid accumulation in macrophages favors bacterial latency by the reversible blockage of bacterial division (33). The preferential persistence of *Brucella* in lipid rich cells suggests that it could be informative to more deeply analyze the relationship between the *Brucella* cell cycle and the availability of lipid nutrients.

In summary, our results allow us to better define the phenotype of the main host cell type favoring the persistence of *B. melitensis* in the spleen of susceptible mice. These data could help to develop new strategies to control *Brucella* infections.

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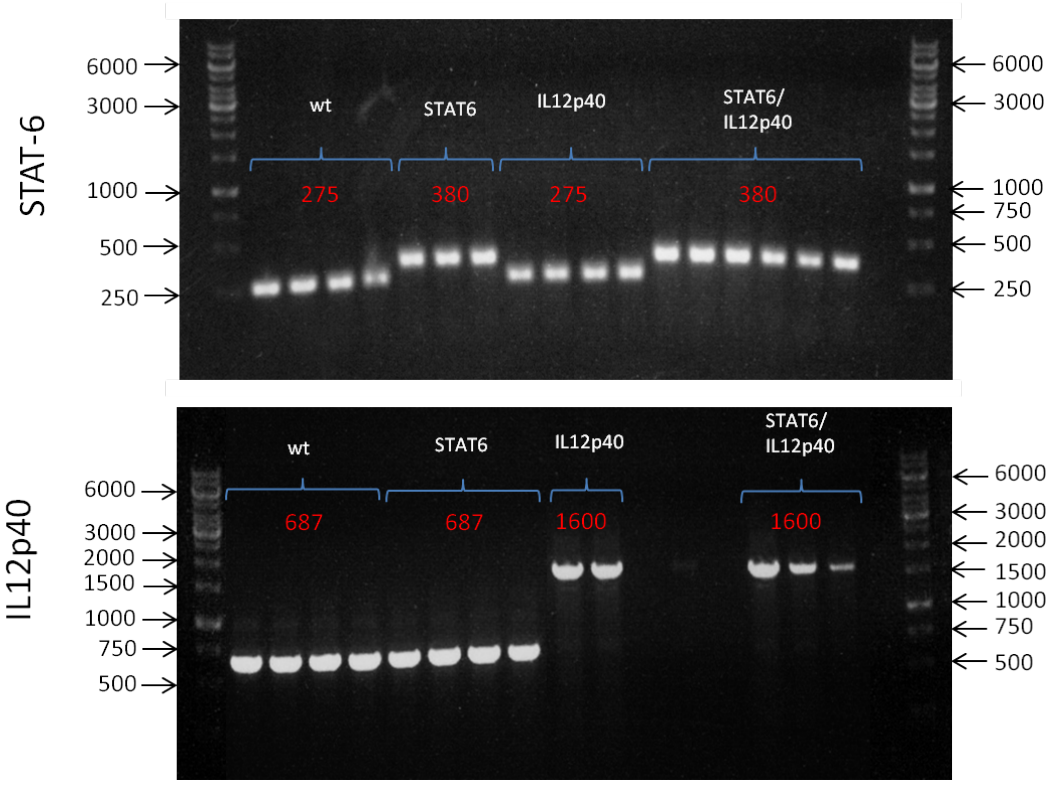

Figure S1: Confirmation of the deficiency of IL12p40 and STAT6 in BALB/c mice used for the experiments. Data are the PCR products of mice DNA on agarose gel. **A,** PCR amplification of STAT6 gene with the lanes from the left to the right being: Ladder, DNA of wt, STAT6-deficient mice, IL12p40-deficient mice, STAT6/IL12p40-deficient mice, ladder. The amplification in Wt and IL12p40-deficient mice is 275 base pairs (bps) long and the amplification in STAT6 and STAT6/IL12p40-deficient mice is 380 bps long. **B,** PCR amplification of IL12p40 gene with the lanes from the left to the right being: Ladder, DNA of wt mice, STAT6-deficient mice, IL12p40-deficient mice, STAT6/IL12p40-deficient mice, ladder. The amplification in Wt and STAT6-deficient mice is 687 base pairs (bps) long and the amplification in IL12p40 and STAT6/IL12p40-deficient mice is about 1600 bps long.

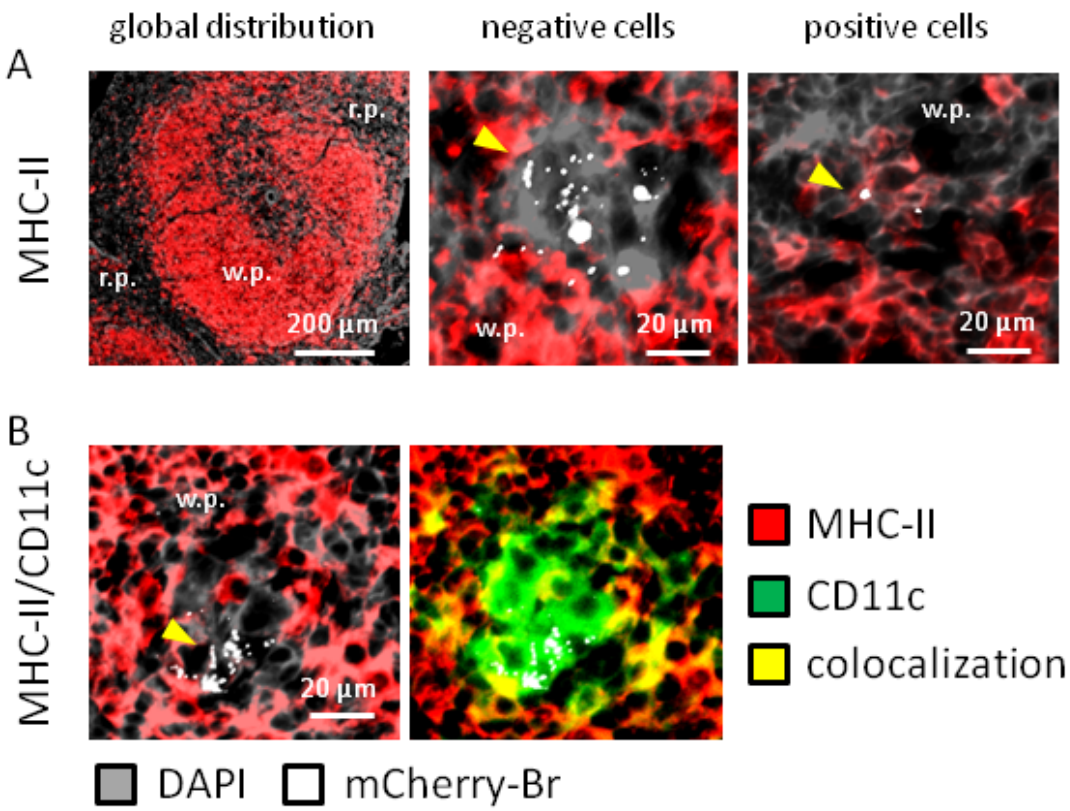

Figure S2: Characterization of MHCII and CD11c infected cells in the spleen of BALB/c IL12p40-deficient mice. IL12p40-deficient BALB/c mice were injected i.n. with 2.10^7 CFU of mCherry-Br. Mice were sacrificed at 28 days post-infection and spleens were collected and examined by immunohistofluorescence..The upper panels show global distribution of MHCII-expressing cells in the spleen (left), mCherry-Br colocalization with negative cells for MHCII (middle) and mCherry-Br colocalization with cells weakly expressing MHCII (right). The panels below show mCherry-Br colocalization with negative cells for MHCII (left) and colocalization of mCherry-Br and CD11c-expressing cells negative for MHCII (right). Panels are color-coded with the text for phalloidin, the antigen examined or mCherry-Br. Scale bar=200 and 20 µm, as indicated. r.p. : red pulp. w.p.: white pulp. Yellow arrowheads indicate the presence of bacteria. Data are representative of at least two independent experiments.

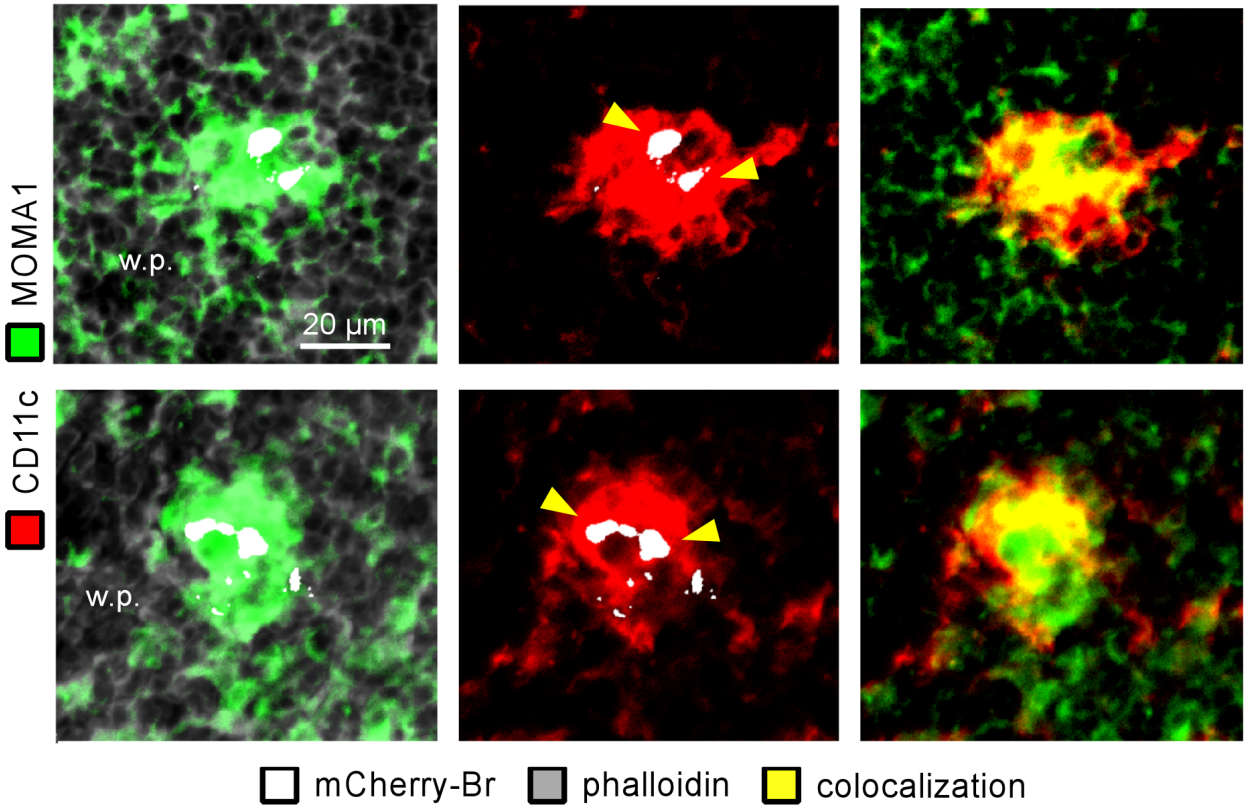

Figure S3: Colocalization between MOMA-1-expressing cells, CD11c-expression cells and mCherry-Br staining in the spleen of BALB/c IL12p40-deficient mice. IL12p40-deficient BALB/c mice were injected i.n. with 2.10^7 CFU of mCherry-Br. Mice were sacrificed at 28 days post-infection and spleens were collected and examined by immunohistofluorescence. The left panels show mCherry-Br colocalizarion wirh MOMA-1-expressing cells, the middle panels show mCherry-Br colocalization with CD11c-expressing cells and the right panel shows colocalization of MOMA-1- and CD11c-expressing cells. Panels are color-coded with the text for phalloidin, the antigen examined or mCherry-Br. Scale bar= 20 µm, as indicated. w.p.: white pulp. Yellow arrowheads indicate the presence of bacteria. Data are representative of at least two independent experiments.

DISCUSSION AND CONCLUSIONS

1. Context

Brucella is responsible for a particularly pernicious disease in livestock all over the world. Abortion in females occurs only once after a long latency of bacteria during which animals generally do not show any visible symptoms. In males, the pathogen can also cause epididymitis and orchitis resulting in sterility. These symptoms are not specific and farmers are not always conscious that *Brucella* is the cause, but economic losses can be huge. As soon as the diagnosis is made, the only way to stop the disease in a herd is to kill all the animals that have been in contact with the infected ones. Moreover, wild animals constitute a reservoir and can transmit the bacterium to domestic animals. Vaccination can reduce the risk. Current vaccines are able to prevent transmission of the pathogen in the herd but are not optimal since they can trigger abortion and interfere with the diagnosis. Moreover, these vaccines are not safe enough to be used in humans. Throughout the last years, scientists have improved their knowledge of *Brucella* including its establishment in the host cells and the mechanisms used to hide from the immune system and to manipulate the host. However, interactions of the pathogen with the immune system are not yet fully understood and mechanisms used by *Brucella* to establish a long lasting infection are far from being elucidated. We performed this work to contribute to the knowledge of this exciting pathogen and the associated host immune response, hoping also to improve the understanding of other intracellular pathogens that could have similar infectious mechanisms.

2. Immune response against Brucella melitensis following intranasal infection

Much of the information acquired concerning *Brucella* infections and the associated host immune response has been obtained using the intraperitoneal model of infection in mice. Our work uses an intranasal (i.n.) model and shows that the route of infection is an important parameter that will influence the outcome of experimental murine infections. Dissemination and localization can be different. Cell populations can change depending on the location, and classes of effector mechanisms can be distinct (Barrigan *et al.*, 2011).

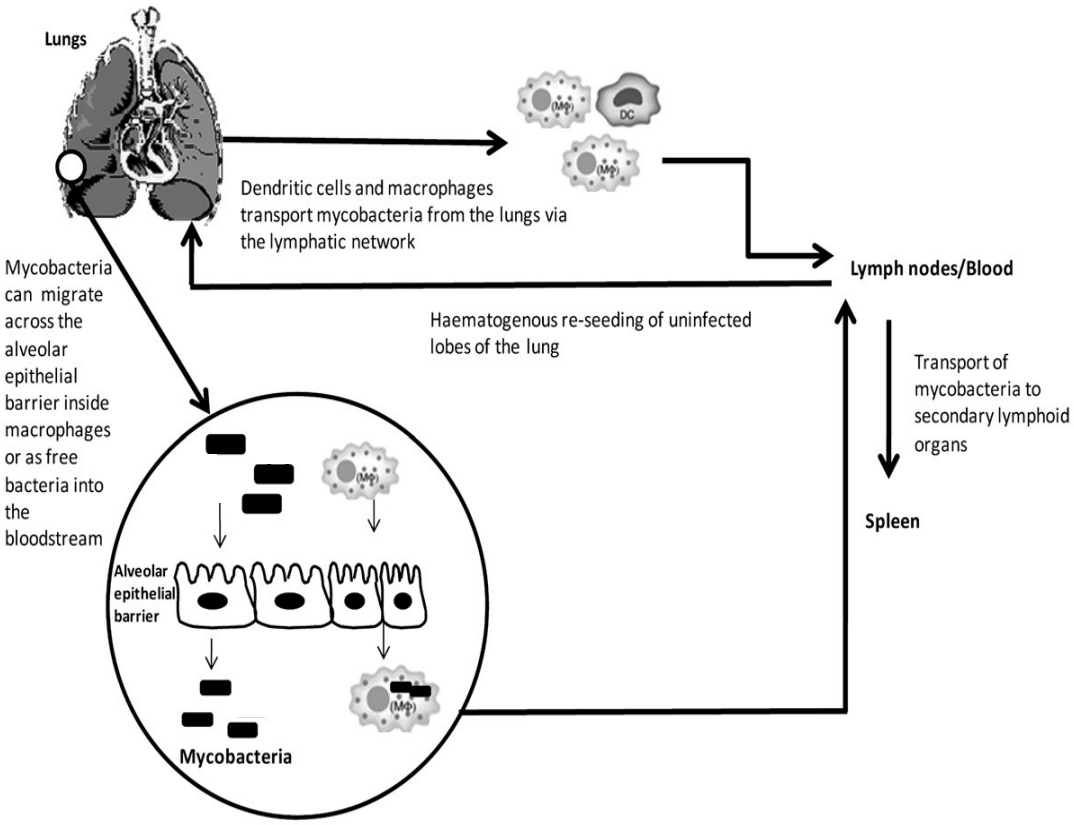

Figure I: Migration pathways of *Mycobacterium tuberculosis* from the primary site of infection to the lymph nodes and the bloodstream (source: adapted from Krishnan et al, 2010).

2.1. Influence of the model of infection on dissemination and localization of bacteria

Ten minutes after an i.p. infection in mice, the blood, spleen and liver are colonized. This systemic dissemination is drastically reduced in i.n. infection, where the respiratory tract and mesenteric lymph nodes are directly colonized (Archambaud et al, 2010), but 5 days are necessary before bacteria are detected in the spleens and livers of all of the infected mice. Bacteria are not detected in the blood of wild-type mice infected by the i.n. route. However, detection of bacteria in the blood of IL-12p35 and IFNγ knockout mice allows us to speculate that *Brucella* disseminate progressively from the lungs to other organs via the blood. The exact pathway used by the pathogen to exit the respiratory tract is not known, but it is known that the brucellae are phagocytosed by alveolar macrophages (Archambaud, 2010) and these cells as well as DCs can transport the bacteria to draining lymph nodes (Archambaud, 2010). Figure I shows pathways potentially used by *Mycobacterium tuberculosis* to disseminate in the host (Figure 1). Free or intracellular bacteria can be released from the lungs directly into the bloodstream by migration across alveolar epithelial barriers. Alternatively, they may migrate through the lymph to the draining lymph nodes thanks to dendritic cells and macrophages. Dissemination routes could be similar for *Brucella*.

Differences observed in *Brucella* dissemination to the deep organs between the i.p. and i.n. routes of infection could explain why bacteria appear everywhere in the spleen, including the red pulp, during the first days after an i.p. infection, and not after an i.n. infection where *Brucella* appear mainly in the white pulp. When foreign substances or pathogens reach the spleen, they are usually stopped and phagocytozed by macrophages in the marginal zone. The large amount of *Brucella* reaching the spleen after an i.p. inoculation could be too large to be contained in the marginal zone and the excess could spread to the red pulp.

2.2. Influence of the model of infection on the immune effectors

In previous i.p. studies in our lab, it has been confirmed that production of IL-12 and presentation of *Brucella* antigens with the MHCII complex are important for triggering a protective Th1 immune response. IFNγ-producing $CD4^+$T lymphocytes are the crucial population to control *B. melitensis* during the primary infection. In the same model, Th2 and IFNγ-producing $CD8^+$T lymphocytes do not play major roles in effective defense against the pathogen (Vitry et al, 2012). With the i.n. model, we obtained similar results for these cellular immune responses and we showed that the Th1 pathway is also the crucial response for controlling the infection.

However, analysis of the lungs, spleen and liver following intranasal infection allowed us to improve our knowledge on the role of IL-23, a cytokine generally associated with the Th17 immune response. IL12p19 (IL-23)-deficient mice are not susceptible as long as the Th1 immune response is functional, but the cytokine becomes important in the absence of this response. It is true for the i.p. model as well as for i.n. model: IL-12p35 (IL-12)-deficient mice have a higher bacterial load than wt mice in the spleen, but less than IL-12p40 (IL-12 and IL-23)-deficient mice. However, the importance of IL-23 is accentuated in the i.n. model because we were able to observe that IL-12p35-deficient mice, but not IL-12p40-deficient mice, eliminate the bacterium from the liver and lungs in the long term. This observation also showed that elimination of bacteria depends on the organ involved.

Differences in effectors have been highlighted between the i.n. model and the i.p. model. Intraperitoneally infected IL-17Ra knockout mice are not more susceptible than infected wt mice. On the contrary, IL-17Ra is important for controlling the bacterial load in the lungs of intranasally infected mice during the first month p.i.. IL-23 is usually necessary to induce IL-17 production, but in our study, IL-17a production does not seem to be linked to the presence of IL-23 because, as explained above, IL-23-deficient mice are not more susceptible than wt mice. IL-23-independent induction of IL-17 has already been reported (Hasegawa et al, 2013). These authors showed that IL-1β could act as a substitute for IL-23 to induce the cytokine. In our model, IL-1β-deficient mice are not susceptible, but the two cytokines could have redundant functionality.

γδ T lymphocytes are particularly abundant cells at mucosal surfaces (Martin et al, 2009), they are potential early producers of IL-17 and IFNγ (Roark et al, 2008), and they can play a protective role against intracellular pathogens invading the mucosal environment, such as *Listeria monocytogenes* or *Mycobacterium bovis* (Sheridan et al, 2013; McGill et al, 2014). This is why we tested deficient mice for this cell population. It turns out that they actually play a role in the control of the bacterial load during the first 12 days of infection. The reason for their positive role in protection has not been determined: in contrast to $CD4^+$T cells, γδ T lymphocytes do not seem to produce IFNγ during the infection, and we do not detect IL-17a production by flow cytometry during the infection. The level of this last cytokine stays perhaps too low to observe a signal or its production could increase at other time points. Other cell types can also produce IL-17 (Veldhoen et al, 2006; Cua et al, 2010) and thus could be contributing to resistance to *Brucella* infection in the i.n. model.

With the i.p. model, primary infection is not controlled by B lymphocytes but in case of a secondary infection, specific antibodies generated in response to the first contact with the pathogen become essential effectors to control the challenge strain in the blood and, subsequently, to limit dissemination to the spleen. Primary i.n. infection is also not controlled by B lymphocytes, but the infection triggers production of specific antibodies. However, in this case they do not play a major role in controlling the secondary i.n. infection because most mice completely eliminate the challenge strain from the spleen even in absence of B cells (MuMT-deficient mice). After i.p. infection of mice, we have shown that bacteria reach deep organs extracellularly through blood during the first day of infection (Vitry et al,2014). This may explain the importance of antibodies in eliminating the invaders. We speculate that after an i.n. infection, bacteria are phagocytozed by host cells and progressively leave the lungs and/or lymph nodes mainly inside cells; this is probably why antibodies are not playing a major role in the elimination of the bacteria. However, it is important to note that the group of Zinkernagel has shown that muMT-deficient mice can still produce immunoglobulin A (Macpherson *et al.*, 2001). Consequently, we looked for specific IgA in serum and broncho-alveolar lavage by ELISA but we could not detect this immunoglobulin in these mice (data not shown).

2.3. Role of IFNγ in the survival of infected mice

Whatever the model of infection, the presence of IFNγ is essential for the survival of infected mice. If IFNγ is produced by CD4+ T cells, the control of the bacterial load is better than if it is produced by other cell types, but as long as it is present, mice survive the infection. IFNγ is usually considered as a cytokine that triggers the production of effector mechanisms essential for bacterial control, and its absence would prevent control of bacteria leading to death of mice. However, the explanation for its role in the survival of mice during experimental *Brucella* infections seems to be more complex. It is true that IFNγ-deficient mice have a higher bacterial load than wt mice, but CD3-deficient mice do not die and they have as many bacteria in their organs as IFNγ-deficient mice. This means that failure to control replication of the brucellae does not seem to be sufficient to induce death in mice. But, IFNγ-deficient mice have a higher level of recruitment of neutrophils to their spleens, livers and lungs in response to *Brucella* infection than do CD3-deficient mice. This can lead to large zones of obstruction in the alveoli. Indeed, IFNγ seems to play a role in hematopoiesis during inflammation, by increasing monopoiesis and inhibiting neutrophil development (de Bruin et al, 2012). And it has been demonstrated that the absence of this cytokine leads to a massive granulocytosis in the spleen and blood of mice infected with *Mycobacterium bovis* (Murray et al, 1998). It is also known that neutrophils can be responsible for tissue destruction during inflammation through secretion of mediators and the release of soluble microbicidals into the external environment to kill pathogens and infected cells (Smith et al, 1994; Wright et al, 2010). Accordingly, systemic inflammation and the activity of granulocytes could be the main cause of death in IFNγ-deficient mice infected with *Brucella*.

3. Reservoir cells for B. melitensis during the chronic phase of infection in a susceptible mouse

Brucella melitensis must be able to reach an intracellular niche favorable for its persistence in the host, but the phenotype of the host cells that facilitate its persistence after the acute phase of infection has not been elucidated. Unfortunately, study of infected cells by immunohistofluorescence in C57BL/6 wt mice after 5 days is very difficult because the bacterial load is below the limit of detection of 10^6 CFU per spleen. In our lab, we decided to use IL12p40-deficient BALB/c mice that are more susceptible to the infection and have a higher bacterial load in the spleen. Previous work with the i.p. model allowed us to postulate that *Brucella* infects MOMA-1 macrophages close to the marginal zone and CD11c/DEC205 dendritic cells in the center of the white pulp of the spleen 12 days post-infection (Copin et al, 2012). In the current work using the i.n. model, we wanted to study the niche of *Brucella* in the spleen during the chronic phase. In wt mice, *Brucella* are eliminated from the lungs and liver after 20 days but persist in the spleens. Thus, we considered 28 days post-infection as being representative of the chronic phase. BALB/c IL12p40-deficient mice do not develop Th1/Th17 immune responses. Instead, they have a pro-Th2 bias. This means that M1 polarization of macrophages is inhibited and that differentiation into alternatively activated macrophages (M2) is facilitated (Bastos *et al*., 2002). These macrophages are known to constitute a favorable niche for intracellular bacteria (see introduction). Moreover, the paper of the research team of Tsolis (Xavier et al, 2013) shows that M2 macrophages ($Fizz^+$ and $CD301^+$ cells) are induced during chronic infection by *Brucella* and that the bacteria survive and replicate particularly well in M2 macrophages *in vitro*. Based on these findings, it has been hypothesized that M2 macrophages could be the major reservoir in the spleens of these mice. Considering the fact that experimental models show that M2 macrophages can be dependent on the STAT6 signaling pathway (Stein et al, 1992; Mandal et al, 2010), we crossed IL12p40 knockout mice with STAT6-deficient mice to obtain a lineage that would be susceptible to the infection, but that would not have Th2 cytokine dependent M2 polarization. We speculated that these double knockout mice would control better the chronic infection due to the absence of the proposed M2 reservoir.

Surprisingly, enumeration of brucellae in the lungs, spleens and livers after low and high dose infection revealed that double knockout mice are as susceptible as IL12p40 mice. Moreover, analysis of the phenotypes of infected cells at 28 days post-infection in the spleens of both lineages showed that reservoir cells for *Brucella* are similar. This means that reservoir cells are not influenced by the IL-4/IL-13/STAT6 signaling pathway. In fact, many cell types seem to be infected in these conditions but they mainly express CD11c and DEC205, two markers specific for DCs. A fraction of infected cells are also MOMA-1 and ERTR9 positive. This suggests that these cells come from the marginal zone, and migrate to the white pulp in response to the infection, just as MOMA-1$^+$ MMM and ERTR9$^+$ MZM do in response to *Listeria monocytogenes* infection (Jablonska *et al*., 2007). To our knowledge, colocalization between defined markers of distinct cell populations, such as MOMA-1 and CD11c markers or ERTR9 and CD11c markers, has not been described in the spleen. This could be due to acquisition and simultaneous loss of markers during cell migration from the marginal zone to the white pulp after the infection. An alternative explanation could be a technical artifact, due to the superimposition of two distinct cells. Negative or low expression of MHCII on cells infected by *Brucella* is in agreement with a previous *in vitro* study with *B. abortus*, which shows that the bacterium is able to inhibit the expression of this complex to prevent recognition by T cells, evade host immune responses and establish chronic infection (Forestier *et al*., 2000). Infected cells also neither express CD11b nor F4/80. This demonstrates that they are different from the CD11b$^+$ CD11c$^+$ F4/80$^+$ infected inflammatory DCs observed during the acute infection in wild type mice (Copin et al, 2012). We also showed colocalization of mCherry and cells positive for Arg1, but not with Fizz1 and CD301. The absence of these last M2 markers on infected cells and the independence of the phenotype of infected cells from the Stat6 pathway let us speculate that infected cells are not classical M2 macrophages.

Most of the *Brucella* infected cells have high lipid content (Bodipy staining), which is a characteristic usually encountered in 'foamy' macrophages such as those observed during *Mycobacterium tuberculosis* infection (Peyron et al, 2008). Moreover, these foamy macrophages coexpress CD11c, CD205, have a low level of MHCII expression (Ordway et al, 2005) and are positive for Arg1 (Schaale et al, 2013). All of these characteristics are shared with most of cells infected by *Brucella* in our model.

4. Perspectives and Final Conclusions

4.1. Primary immune response

After an intranasal (i.n.) infection of mice with *Brucella melitensis*, bacteria are phagocytozed by alveolar macrophages before being transported to the draining lymph nodes by alveolar macrophages and dendritic cells (Archambaud *et al.*, 2010). The immune system fails to neutralize *Brucella* because of bacterial properties that confer to the pathogen a strong resistance to immune effectors. Escape mechanisms allow *Brucella* to disseminate through the body of its host and establish a replicative niche. Fortunately, bacteria do not remain undetectable by the immune system, and granulomas appear progressively in the liver and spleen to surround infected cells after a week. After 12 days, MHCII-dependent antigen presentation to $CD4^+$ T lymphocytes appears to play a central role in the control of the infection. These T cells begin to produce IFNγ which increases until 50 days p.i.. This pro-inflammatory cytokine induces the production of nitric oxide by innate immune cells that helps control the infection (Copin *et al.*, 2007). This pro-inflammatory immune response developed in the spleen and the liver is sufficient to clear *Brucella* from the liver by one month p.i., but not from the spleen. In the lungs, the immune mechanisms seems to be different; no recruitment is detectable but mucosal γδ T cells and IL-17a production help the Th1 immune response in the early phase of infection. The local immune response seems to be sufficient to eliminate the bacteria which do not persist more than three weeks.

→ Perspectives:

- We have demonstrated that the lungs are infected 3 hours after intranasal infection and that the bacterial load approximately corresponds to the infected dose. At this time point bacteria have not replicated yet. However, infection of the upper respiratory tract is not excluded since the exact number of inoculated bacteria is not known. Analysis of the bacterial load in the upper respiratory tract and the draining lymph nodes would indicate if *Brucella* infects these zones as well as the lower respiratory tract. Utilization of whole body imaging could also be a great method to observe the zones infected by *Brucella* after intranasal inoculation (Hoffman *et al.*, 2007; Gonzalez *et al.,* 2012).

- It would be interesting to study the infection in lymph nodes because we know that mediastinal lymph nodes are rapidly infected after i.n. inoculation in the murin model (Archambaud *et al*., 2010) but the kinetics of these infections have not been investigated. We should analyse the immune response in this organ to compare the protection with the one of lungs, spleen and liver.
- The pathway used by *Brucella* to reach deep organs has not been characterized. With whole body imaging, we could also follow dissemination of the bacteria and perhaps have a clue about the route of spread as has been demonstrated with *Bacillus anthracis* (Glomski et *al.*, 2007).
- We hypothesized that presence of bacteria in the red pulp of the spleen after i.p. infection but not after an i.n. infection could be explained by the rapid dissemination after i.p. infection. *Brucella* could be too abundant to all be trapped by the macrophages of the marginal zone. It has been shown that depletion of MZM and MMM in the spleen of mice impairs trapping of intravenous (i.v.) injected microspheres and *L. monocytogenes* by the marginal zone. They are no more retained and spread to the red pulp (Aichele *et al*., 2003). This experiment could be an indication that if the amount of foreign particles is superior to the number of macrophages in the marginal zone, they would not be retained and reach the red pulp. Injection of high doses of microspheres could allow us to see if a portion of them reaches the red pulp.
- In our study, IL-17A production seems to be independent from IL-23 production. It has already been demonstrated that its production can be independent from IL-23 and that IL-1β can substitute for IL-23 to induce IL-17 (Hasegawa *et al*., 2013). Our model shows that IL-1β-deficient mice are also not susceptible to the infection. It would be interesting to use double knockout IL-23/IL-1β mice in order to see if these two cytokines have redundant activities. Moreover, other cytokines such as IL-6, IL-18 and TGFβ have also been implicated in promoting IL-17 production (Veldhoen *et al*., 2006; Lalor *et al*., 2011). Their depletion in mice could also give answers.
- IL-17A participates in control of infection in the lungs, but we were not able to detect production of this cytokine by flow cytometry. RT-PCR or restimulation of cells could be alternatives to quantify IL-17A production.

- We have tested the function of γδ T cells in the lungs during *Brucella* infection because they are abundant in the mucosal environment, are important for the control of infections in these zones, and are potential producers of IL-17 and IFNγ. But other cell types, such as Natural Killer cells, NK T cells and innate lymphoid cells (ILCs), are also able to produce these cytokines and participate in mucosal immunity. Depletion of these cell types in mice is also a possibility to enlarge our knowledge on cell populations playing a role in the control of i.n. infection.
- The reason why IFNγ-deficient mice die was investigated as a side project during the thesis. Massive inflammation due to neutrophil recruitment was observed. Reduction of inflammation by an anti-inflammatory treatment could be planned to thwart the deleterious effects of inflammation. However, we have to keep in mind that inhibition of global inflammation could also be detrimental for mice. Recently, mice genetically deficient for neutrophils (*Genista* mice), have been generated (Ordonez-Rueda *et al*., 2012). Crossbreeding between these mice and IFNγ-deficient mice could generate double knockout mice that would not die if inflammation due to neutrophilia were responsible for the death of mice. An alternative is the utilization of Ly6G-specific antibody (1A8) to deplete neutrophils (Daley *et al.*, 2008). Dr. Muraille has tested this antibody once in a model of footpad infection, but he did not observe a reduction of neutrophils in the lesion. We could nevertheless try the antibody in our model with different kinetics of injection and different doses.
- IFNγ is essential for the survival of mice but it apparently has to be produced by $CD4^+$ T cells for efficient control of the infection. Without this IFNγ-producing population (Th1), mice are more susceptible despite the fact that other cell types, such as $CD8^+$ T cells or NK T cells, are able to produce this cytokine. This suggests that these $CD4^+$ T cells have another function that is particularly effective for controlling the infection. Th1 cells not only produce IFNγ, so the expression of other cytokines such as IL-2 and TNFα (Darrah et al., 2007) could also be studied to solve this mystery.

4.2. Reservoir cells

After one month p.i. , the spleen of wt mice is the last studied organ that is still infected. This suggests that it contains a particularly favorable niche where *Brucella* can persist for the long term. We had to investigate the question of the reservoir cells by using susceptible IL12p40-deficient mice because of the insufficient amount of bacteria in the spleen of wt mice. In this model, *Brucella* is able to infect many cell types but its main sanctuary is apparently similar to the 'foamy' macrophages infected during *Mycobacterium tuberculosis* persistence. These cells are CD11c+ CD205+ Bodipy+ Arg1+ and MHCII low/neg and could constitute a niche with a nutrient source for the pathogen. The fact that deletion of STAT6 in IL12p40-deficient mice does not influence the infected cell types in the spleen and that they do not express M2 markers such as CD301 and Fizz let us hypothesize that these cells are not classical M2 macrophages. It also seems that infected MMM and MZM migrate from the marginal zone to the white pulp because of the expression of MOMA-1 and ERTR9 on a portion of the infected cells.

→ Perspectives:

- *Brucella* are able to infect different cell types *in vitro* but our results show that one cell type is mainly infected, or at least, different cell types that share common markers. It could be interesting to target one of these markers with liposomes; composed of a phospholipid membrane and an encapsulated aqueous phase, these structures can contain a therapeutic agent (Sofou *et al.*, 2008). We could conjugate liposomes with a specific antibody (for example DEC205 antibody) on their surface to interact with the particular cell population positive for the marker (DEC205). Once on the targeted cells, liposomes can fuse with the cellular membrane or be internalized by endocytosis to deliver the drug. This approach could reduce or even eliminate persistence of the bacterium in the spleens of mice.
- We were not able to characterize reservoir cells in wild type mice because of the limit of detection (10^6 CFU/spleen), but targeting of these cells with liposomes in wt and IL12p40-deficient mice could permit us to see if persistence is reduced in the same way between the two lineages and could indicate if the reservoir cells are identical.

- We are not able to explain why mCherry-Br colocalizes with markers usually located on different cell types, such as MOMA-1 and DEC205. We hypothesized that it is due to infection and migration of cells: during migration, infected cells could acquire new markers and lose others. However, it is not excluded that it is a technical artifact. Use of confocal microscopy could help to see if markers are only present on one cell or if there is an overlap of two cell types.
- Study of the reservoir cell at later times would tell us if *Brucella* stays in the same cell type during its persistence. In the susceptible mice used for our experiments, CFUs slightly decrease at 50 days p.i., but observation of infected cells remains possible.

4.3. Secondary immune response

Upon secondary i.n. infection, the experimented immune system of the mouse is able to fight *Brucella* with a stronger specific response. But contrary to the i.p. model of infection, specific circulating antibodies secreted by B lymphocytes do not play a major role in the clearance of the challenge strain. It seems that effector memory T lymphocytes are sufficient to coordinate bacterial clearance and provide a long-term protection in organs. The intracellular location of *Brucella* during systemic dissemination is probably the reason why cell-mediated immune response is the only protective immune response against a second invasion by *Brucella melitensis*.

→ Perspectives

- We have only investigated the humoral immune response in the control of secondary infection, but study of the cellular immunity should clearly be interesting. It is known that primary and secondary immune effectors can be different (Kirby *et al.,* 2002). This would improve our knowledge of the protective immune response following a challenge and could help to generate an effective vaccine.

4.4. Conclusion

Taken together, our results improve our knowledge of the host immune response to brucellosis. They highlight the importance of the model of infection and show that the analyzed organ can influence the outcome. Our data also provide insight into the identity of the reservoir cells inhabited by the brucellae during the chronic phase of infection in susceptible mice. These findings could help to define an effective treatment by a specific target of infected cells. Finally, differences observed between i.p. and i.n. model of a secondary infection could help to define a rational strategy for the generation of new vaccines against brucellosis. This thesis has laid a cornerstone in the building of the knowledge on *Brucella* and the associated immune response but it has also shown that there remains a lot of work in the future to elucidate the complete protective immune response against *Brucella* and the mechanisms used by this pathogen to infect its host with such efficiency.

APPENDIX

1. In Situ Microscopy Analysis Reveals Local Innate Immune Response Developed around Brucella Infected Cells in Resistant and Susceptible Mice

Richard Copin, Marie-Alice Vitry, Delphine Hanot Mambres, Arnaud Machelart, Carl De Trez, Jean-Marie Vanderwinden, Stefan Magez, Shizuo Akira, Bernhard Ryffel, Yves Carlier, Jean-Jacques Letesson, Eric Muraille

2. *Humoral Immunity and CD4+ Th1 Cells Are Both Necessary for a Fully Protective Immune Response upon Secondary Infection with Brucella melitensis*

Marie-Alice Vitry, Delphine Hanot Mambres, Carl De Trez, Shizuo Akira, Bernhard Ryffel, Jean-Jacques Letesson and Eric Muraille

3. *Brucella melitensis Invades Murine Erythrocytes during Infection*

Marie-Alice Vitry, Delphine Hanot Mambres, Michaël Deghelt, Katrin Hack, Arnaud Machelart, Frédéric Lhomme, Jean-Marie Vanderwinden, Marjorie Vermeersch, Carl De Trez, David Pérez-Morga, Jean-Jacques Letesson and Eric Muraille

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